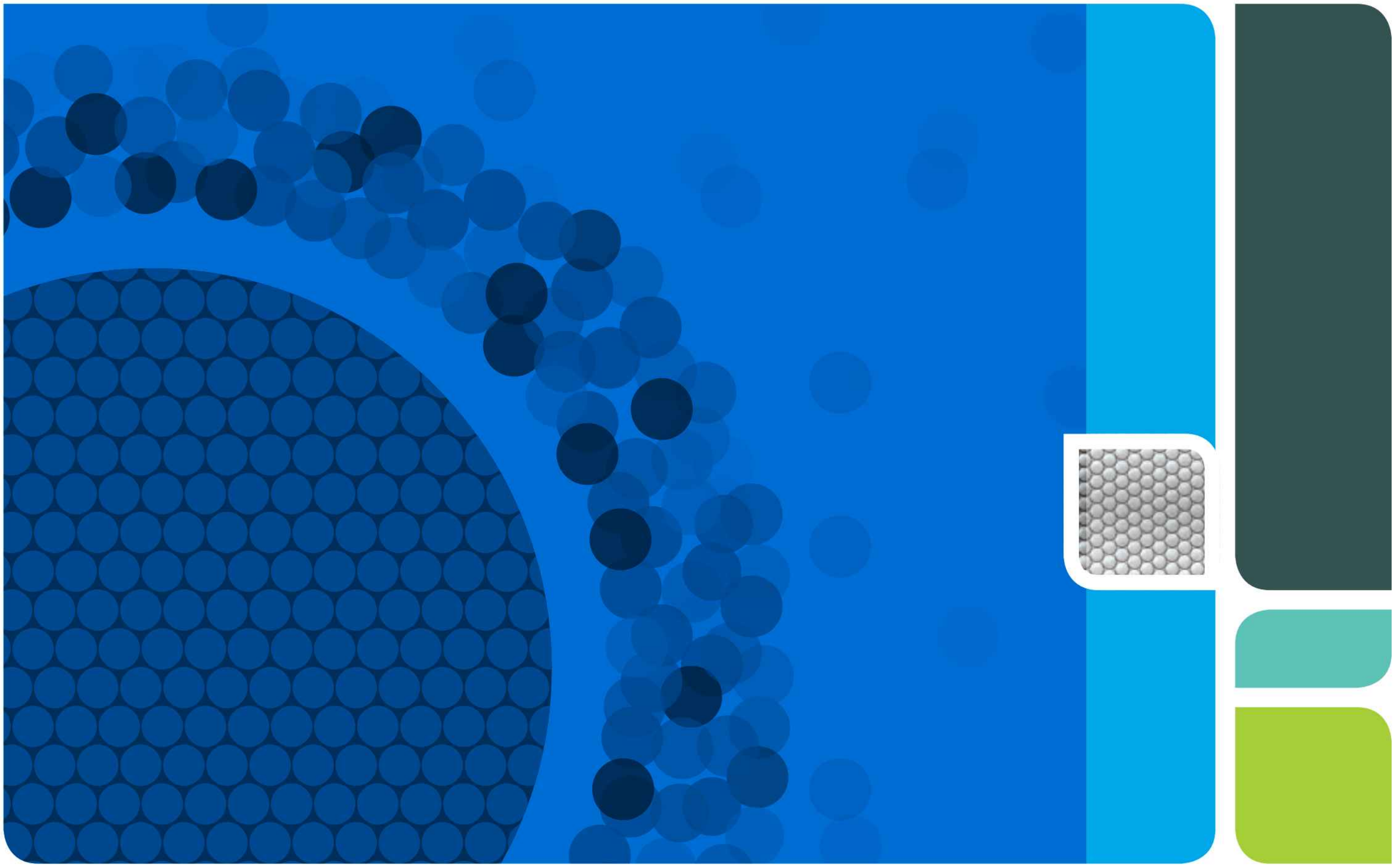
Droplet Digital™ PCR



# Droplet Digital™ PCR
## Applications Guide

BIO-RAD

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# 1 Droplet Digital™ PCR

## Introduction

Droplet Digital polymerase chain reaction (ddPCR™) was developed to provide high-precision, absolute quantification of nucleic acid target sequences with wide-ranging applications for both research and clinical diagnostic applications. ddPCR measures absolute quantities by counting nucleic acid molecules encapsulated in discrete, volumetrically defined water-in-oil droplet partitions. Droplet Digital PCR using Bio-Rad's QX100™ or QX200™ Droplet Digital PCR system overcomes the previous lack of scalable and practical technologies for digital PCR implementation.

ddPCR has the following benefits for nucleic acid quantification:

- Unparalleled precision — the massive sample partitioning afforded by ddPCR enables small fold differences in target DNA sequence between samples to be reliably measured
- Increased signal-to-noise — enrich for rare targets by reducing competition that comes from high-copy templates
- Removal of PCR efficiency bias — error rates are reduced by removing the amplification efficiency reliance of PCR, enabling accurate quantification of targets
- Simplified quantification — a standard curve is not required for absolute quantification

## QX100/QX200 Workflow

Bio-Rad's QX100 or QX200 ddPCR System (Figure 1.1) combines water-oil emulsion droplet technology with microfluidics. The QX200 Droplet Generator partitions samples into 20,000 droplets (Figure 1.2). PCR amplification is carried out within each droplet using a thermal cycler. After PCR, droplets are streamed in single file on a QX200 Droplet Reader, which counts the fluorescent positive and negative droplets to calculate target DNA concentration.

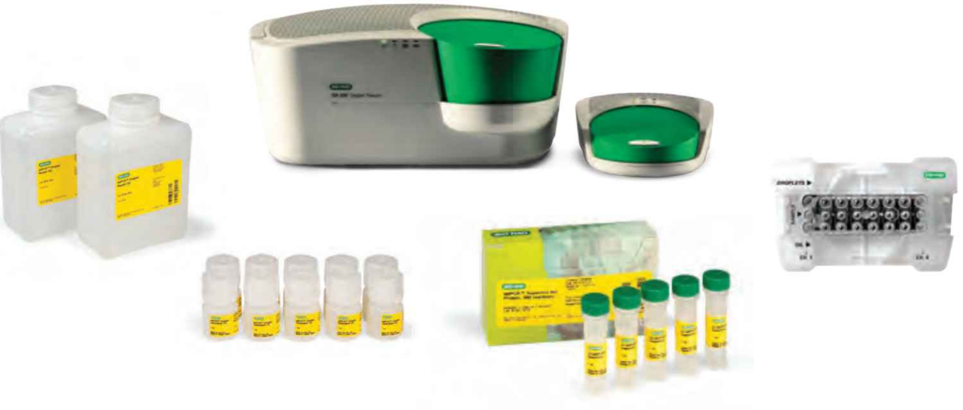


Fig. 1.1. QX200 ddPCR System with associated consumables.

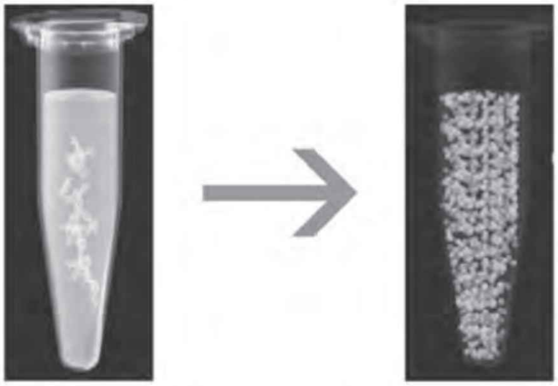


Fig. 1.2. In ddPCR, a single PCR sample is partitioned into 20,000 discrete droplets.

## Droplet Generation

Before droplet generation, ddPCR reactions are prepared in a similar manner as real-time PCR reactions that use TaqMan hydrolysis probes labeled with FAM and HEX (or VIC) reporter fluorophores, or an intercalating dye such as EvaGreen®.

ddPCR must be performed with the proprietary reagents developed specifically for droplet generation by Bio-Rad. Reagent mixes include the ddPCR Supermix for Probes and QX200™ ddPCR™ EvaGreen® Supermix to partition DNA, and the One-Step RT-ddPCR Advanced Kit for Probes.

Samples are placed into a QX100 or QX200 Droplet Generator, which uses specially developed reagents and microfluidics to partition each sample into 20,000 nanoliter-sized droplets. As shown in Figure 1.3, target and background DNA are distributed randomly into the droplets during the partitioning process.

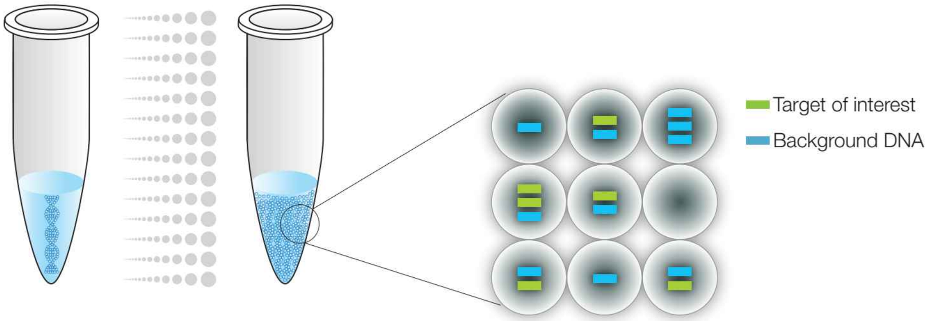


**Fig. 1.3. In ddPCR, a single PCR sample is partitioned into 20,000 droplets.**

Droplet generation produces uniform droplets for the sample, enabling precise target quantification (Figure 1.4).

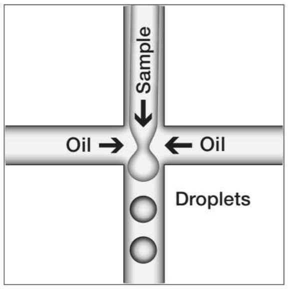


**Fig. 1.4. The droplets created by the QX200 Droplet Generator are uniform in size and volume.**

## PCR Amplification

Droplets are transferred to a 96-well plate for PCR in a thermal cycler. We recommend the C1000 Touch™ Thermal Cycler with 96–deep well reaction module for PCR (Figure 1.5). This high-performance thermal cycler has excellent temperature uniformity and settling across all 96 wells to help ensure successful PCR.

**Fig. 1.5. The C1000 Touch Thermal Cycler provides robust performance for ddPCR experiments.**

## Droplet Reading

Following PCR amplification of the nucleic acid target in the droplets, the plate containing the droplets is placed in a QX100 or QX200 Droplet Reader, which analyzes each droplet individually using a two-color detection system (set to detect FAM and HEX [or VIC]).

The autosampler of the droplet reader picks up the droplets from each well of the PCR plate (Figure 1.6).

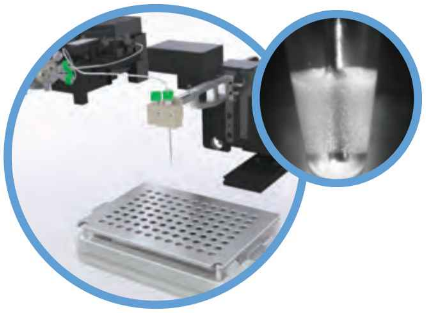


**Fig. 1.6. QX200 Droplet Reader autosampler.**

Droplets are spaced out individually for fluorescence reading by the droplet reader (Figure 1.7). Fluorescence in two channels is then measured for individual droplets.

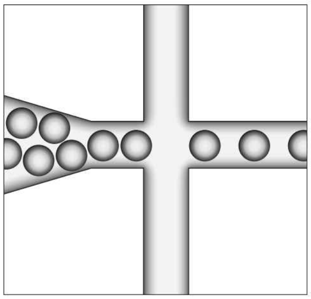


**Fig. 1.7. Separating individual droplets in the QX100 Droplet Reader.**

Positive droplets, which contain at least one copy of the target DNA molecule, exhibit increased fluorescence compared to negative droplets (Figure 1.8).

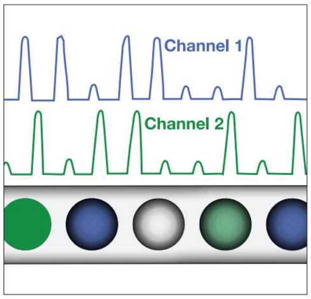


**Fig. 1.8. Fluorescence readings are measured for each droplet in two channels.**

## ddPCR Data Analysis

Droplet Digital PCR data can be viewed as a 1-D plot with each droplet from a sample plotted on the graph of fluorescence intensity vs. droplet number. In Figure 1.9, all positive droplets, those above the red threshold line, are scored as positive and each is assigned a value of 1. All negative droplets, those below the red threshold line, are scored as negative and each is assigned a value of 0.

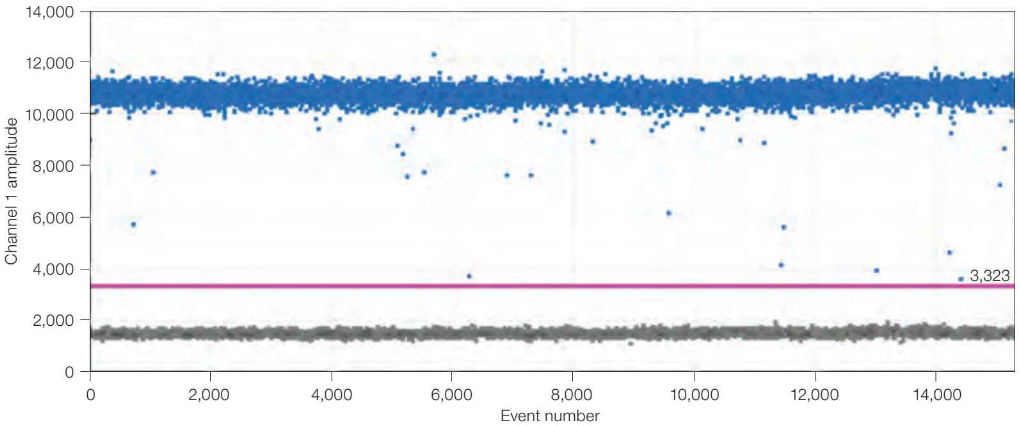


**Fig. 1.9. Each droplet from a sample is plotted on the graph of fluorescence intensity vs. droplet number.**

Droplet Digital PCR data from a duplex experiment in which two targets are PCR amplified can also be viewed in a 2-D plot in which channel 1 fluorescence (FAM) is plotted against channel 2 fluorescence (HEX or VIC) for each droplet (Figure 1.10).

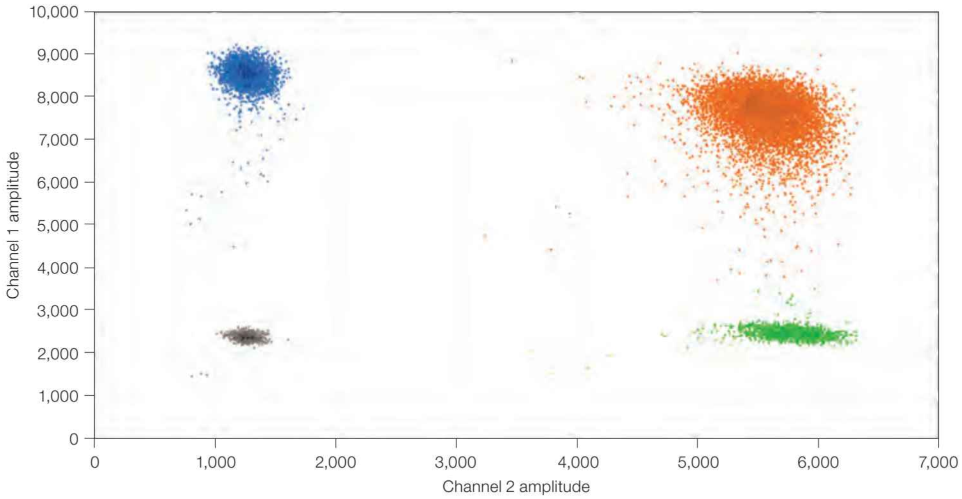


**Fig. 1.10. 2-D plot of droplet fluorescence.**

Because the DNA distribution into the droplets follows a random pattern, droplets cluster into four groups:

- FAM negative, HEX negative (double-negative droplets)
- FAM positive, HEX negative
- FAM negative, HEX positive
- FAM positive, HEX positive (double-positive droplets)

QuantaSoft™ Software measures the number of positive and negative droplets for each fluorophore in each sample. The software then fits the fraction of positive droplets to a Poisson algorithm to determine the starting concentration of the target DNA molecule in units of copies/µl input (Figure 1.11).

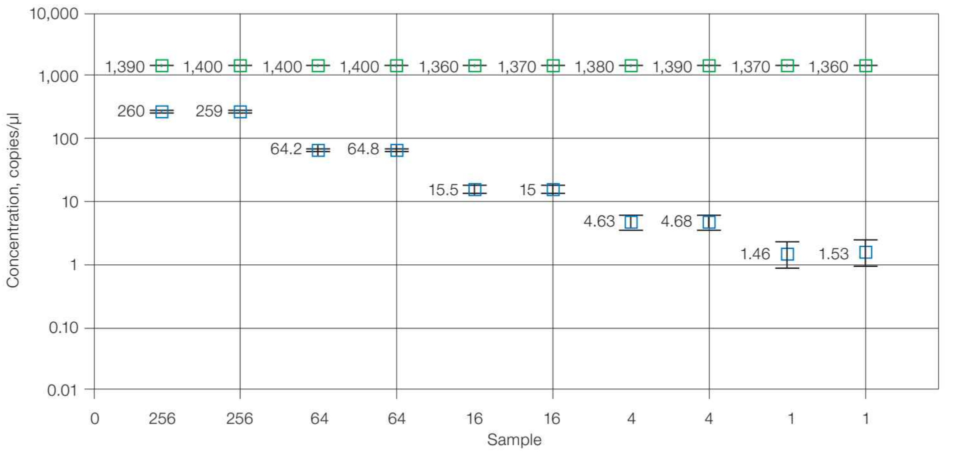


**Fig. 1.11. Sample concentrations are plotted as copies/µl from the sample.**

# Emerging Applications of Droplet Digital PCR

Sample partitioning allows the sensitive, specific detection of single template molecules as well as precise quantification. It also mitigates the effects of target competition, making PCR amplification less sensitive to inhibition and greatly improving the discriminatory capacity of assays that differ by only a single nucleotide. Digital PCR offers the benefits of absolute quantification and greatly enhanced sensitivity. Therefore, its application in the following areas is growing:

- Absolute quantification — ddPCR provides a concentration of target DNA copies per input sample without the need for running standard curves, making this technique ideal for target DNA measurements, viral load analysis, and microbial quantification
- Genomic alterations such as gene copy number variation (CNV) — CNVs result in too few or too many dosage-sensitive genes responsible for phenotypic variability, complex behavioral traits, and disease. ddPCR enables measurement of 1.2x differences in gene copy number
- Detection of rare sequences — researchers must amplify single genes in a complex sample, such as a few tumor cells in a wild-type background. ddPCR is sensitive enough to detect rare mutations or sequences
- Gene expression and microRNA analysis — ddPCR provides stand-alone absolute quantification of expression levels, especially low-abundance microRNAs, with sensitivity and precision
- Next-generation sequencing (NGS) — ddPCR quantifies NGS sample library preparations to increase sequencing accuracy and reduce run repeats. Validate sequencing results such as single nucleotide polymorphisms or copy number variations with absolute quantification
- Single cell analysis — the high degree (10- to 100-fold) of cell-cell variation in gene expression and genomic content among homogeneous post-mitotic, progenitor, and stem cell populations drives a need for analysis from single cells. ddPCR enables low copy number quantification
- Genome edit detection — ddPCR enables fast, precise, and cost-effective assessment of HDR (Homology directed repair) and NHEJ (Non-homologous end joining) generated by CRISPR-Cas9 or other genome editing tools

# ddPCR for Absolute Quantification and Experimental Considerations

In a typical digital PCR experiment, the sample is randomly distributed into discrete partitions such that some contain no nucleic acid template and others contain one or more template copies. The partitions are PCR amplified to end point and then read using a droplet reader to determine the fraction of positive partitions, from which the concentration is estimated by modeling as a Poisson distribution. The formula used for Poisson modeling is:

Copies per droplet = $-\ln(1 - p)$

where $p$ = fraction of positive droplets.

Droplets are assigned as positive or negative by thresholding based on their fluorescence amplitude. The number of positive and negative droplets is used to calculate the concentration of the target and reference DNA sequences and their Poisson-based 95% confidence intervals (Figure 1.12).

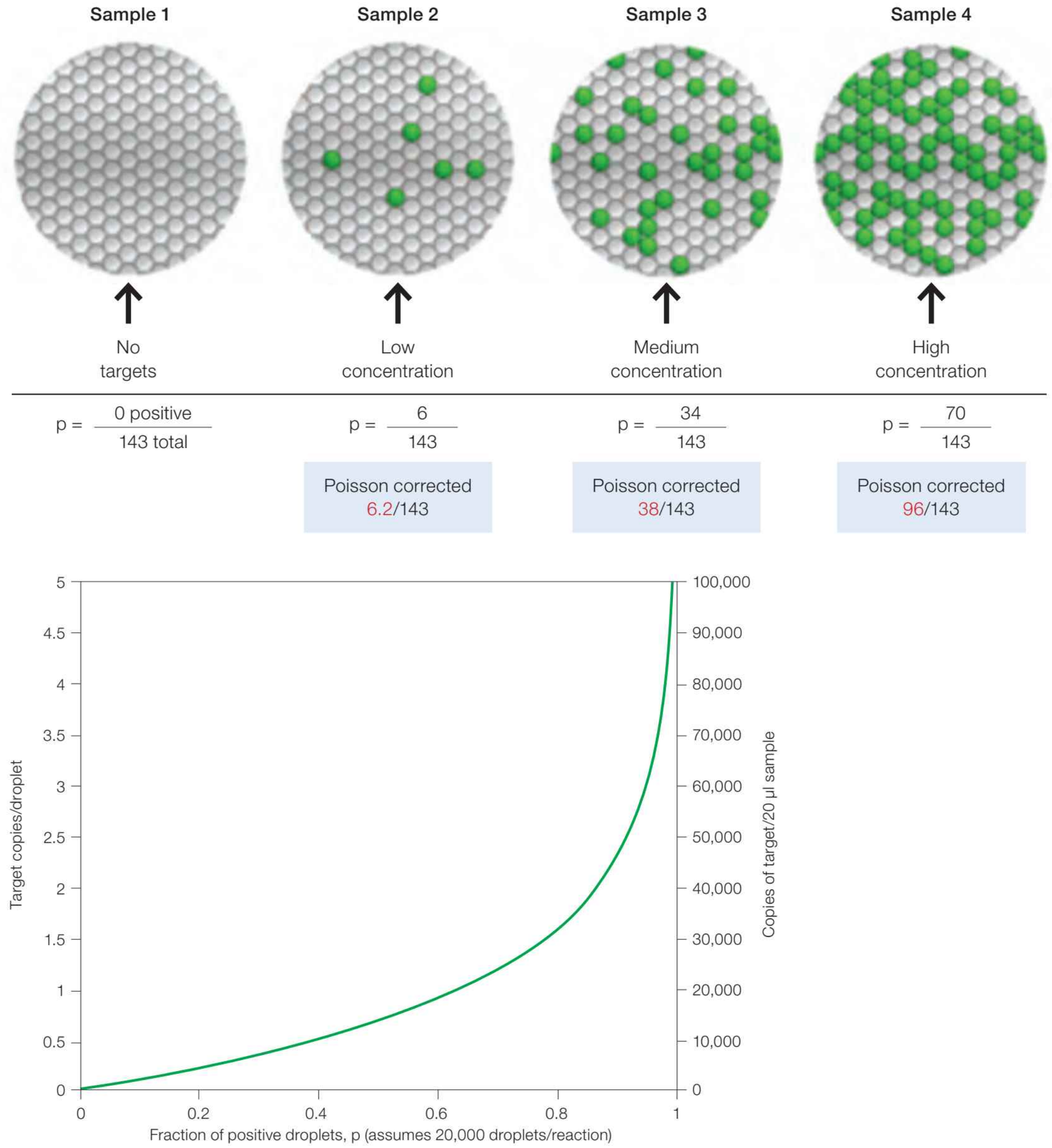


**Fig. 1.12. Estimating target concentration by ddPCR.**

ddPCR is an end-point measurement that enables you to quantify nucleic acids without the use of standard curves and independent of reaction efficiency. The present-or-absent digital format (Figure 1.12) and ability to draw clear thresholds between positive and negative droplet clusters for ddPCR means we now operate in a regime of absolute DNA quantification without the need for external calibrators or endogenous controls. The region separating these clusters is clear, such that the concentration output is not significantly affected by the position of the threshold and results are not biased by the small fraction of droplets that do not reach end point. This is another advantage of conducting many thousand PCR reactions/20 µl sample (Figure 1.13).

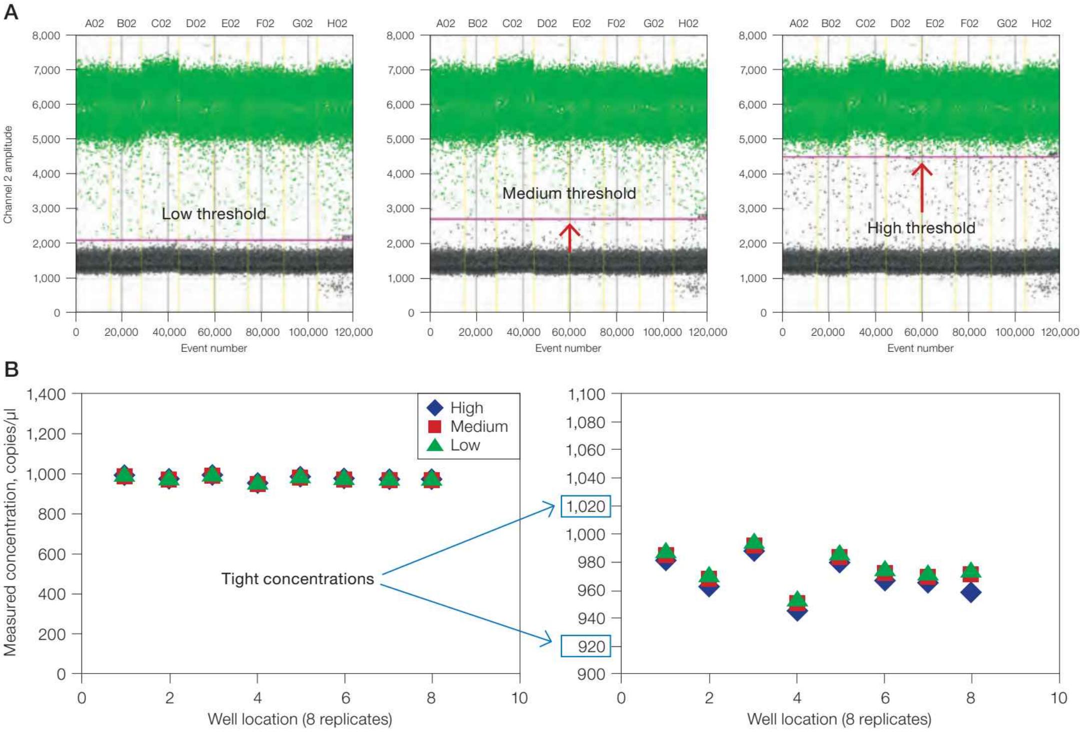


**Fig. 1.13. The power of thousands of replicates using ddPCR. A**, threshold options; **B**, measured concentration for different threshold settings.

Figure 1.13 shows the effect of varying the threshold value (Figure 1.13A) on concentration (Figure 1.13B). Eight ddPCR replicate wells are shown in the plots in Figure 1.13A. Three scenarios are tested for discriminating positive (high amplitude) from negative (low amplitude) droplets and the concentration calls associated with each (Figure 1.13B). A precision of ±5% is maintained regardless of the thresholding method used (see Fig. 1.13B zoomed-in view).

After optimizing the conditions to conduct a ddPCR experiment, the precision and resolving power afforded by partitioning are unprecedented. Figure 1.14A provides an example of this, showing a twofold serial dilution spanning 4 orders of magnitude of *Staphylococcus aureus* template concentration (🟦, FAM channel) in a constant background of human genomic DNA (gDNA) (🟩, VIC channel) run as a duplex assay. Figure 1.14B shows a zoomed-in view that displays the resolution of small copy number differences using ddPCR for a series of 10% dilutions of the same target and reference assays. The error bars represent Poisson 95% confidence intervals.

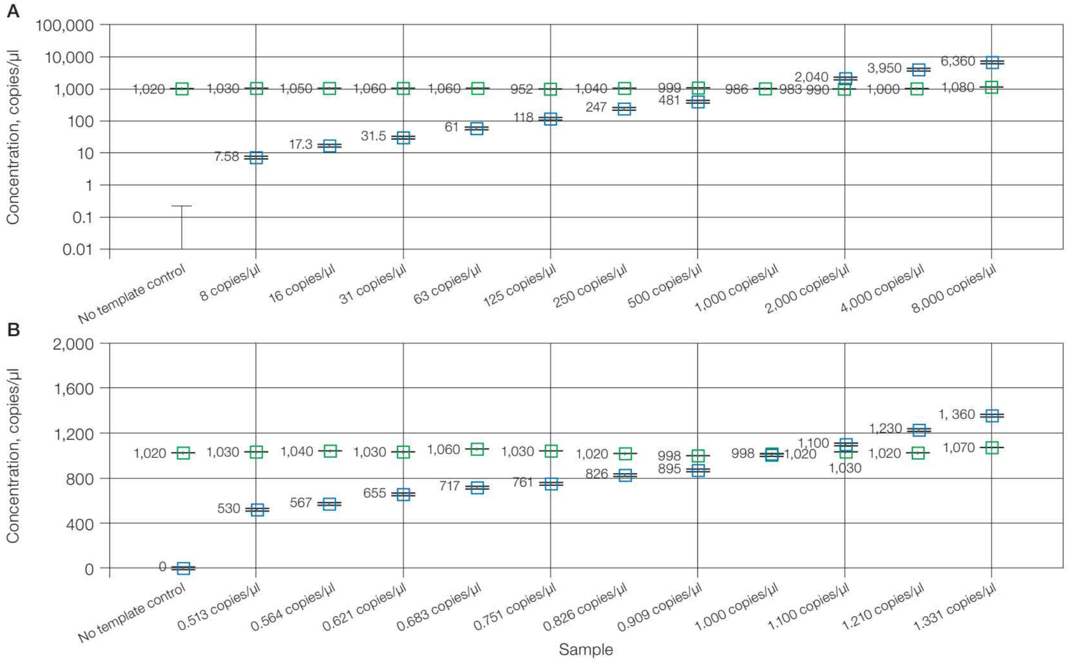


**Fig. 1.14. QuantaSoft Software concentration estimates.** Twofold (**A**) and 10% (**B**) dilution series of *S. aureus* template (🟦) in a constant background of human gDNA (🟩) run as a duplex FAM/VIC assay. The error bars represent Poisson 95% confidence intervals.

# 2 Droplet Digital™ PCR Experiments

## Assay Design for Droplet Digital PCR

As with any PCR-based technology, assay design and sample preparation are important for obtaining high-quality data. Before running a Droplet Digital PCR (ddPCR™) experiment, know the goal or possible expected outcomes of the experiment because different types of experiments require different controls, sample preparation, amounts of DNA or RNA, and data analysis.

The amplification reaction of target molecules in ddPCR workflows follows similar principles of real-time PCR.

- Plan to amplify a 60–200 bp product
- Avoid regions that have secondary structure when possible
- Choose a region that, ideally, has a GC content of 40–60%

### Designing Primers

Widely accepted quantitative PCR (qPCR) design guidelines apply to ddPCR primer design. Important criteria for single primers include melting temperature ($T_m$), length, base composition, and GC content. In addition, because primers are used in pairs, ensure that paired primers do not exhibit significant complementarity between 3' ends because this can result in primer-dimers. Extensive primer-dimer formations can significantly decrease or prevent amplification. The QX200™ Droplet Digital PCR System will support both hydrolysis probe (TaqMan) and DNA binding dye (EvaGreen®) assays. All information in this chapter applies to both types of assay, except for the Designing Probes section.

When designing primers for a target sequence, follow these guidelines:

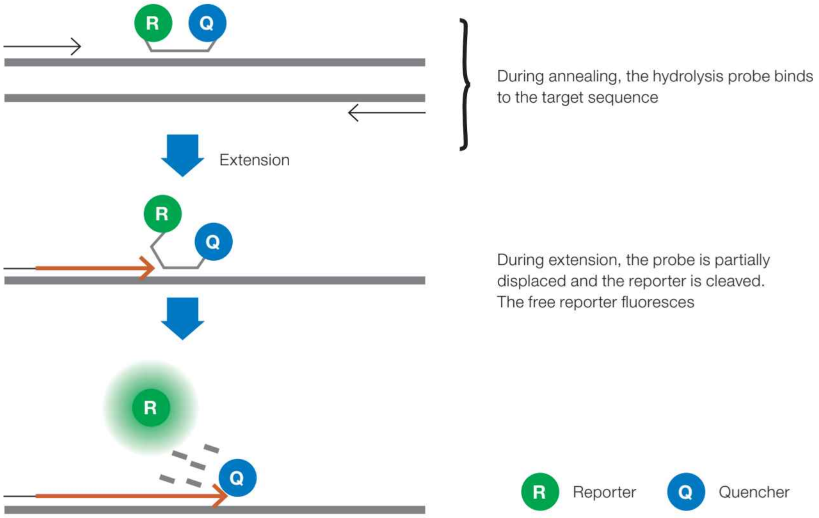
- Design primers that have a GC content of 50–60%
- Strive for a $T_m$ between 50 and 65°C. One way to calculate $T_m$ values is by using the nearest-neighbor method. Use the $T_m$ calculator at **http://www.basic.northwestern.edu/biotools/oligocalc.html**, with values of 50 mM for salt concentration and 300 nM for oligonucleotide concentration
- Avoid secondary structure and adjust primer locations so they are outside the target sequence secondary structure, if required
- Avoid repeats of Gs or Cs longer than 3 bases
- Place Gs and Cs at the 3' nucleotide of primers when possible
- Check forward and reverse primer sequences to ensure no 3' complementarity (avoid primer-dimers)

## Designing Probes

The QX100™ Droplet Digital PCR System is compatible only with TaqMan hydrolysis probes. The QX200 System is compatible with TaqMan hydrolysis probes and EvaGreen® double-stranded DNA (dsDNA) binding dye. Using EvaGreen® or SYBR® Green on the QX100 will damage the system.

Neither the QX100 nor the QX200 System is compatible with SYBR® Green. Advantages of using hydrolysis probes include high specificity, a high signal-to-noise ratio, and the ability to perform multiplex reactions. Hydrolysis assays include a sequence-specific, fluorescently labeled oligonucleotide probe in addition to the sequence-specific primers. TaqMan assays exploit the 5' exonuclease activity of certain thermostable polymerases. The hydrolysis probe is labeled with a fluorescent reporter at the 5' end and a quencher at the 3' end.

When the probe is intact, the fluorescence of the reporter is quenched due to its proximity to the quencher (Figure 2.1). The amplification reaction includes a combined annealing/extension step during which the probe hybridizes to the target and the dsDNA-specific 5' to 3' exonuclease activity of Taq or Tth cleaves off the reporter. As a result, the reporter is separated from the quencher, resulting in a fluorescence signal that is proportional to the amount of amplified product in the sample.



During annealing, the hydrolysis probe binds to the target sequence

During extension, the probe is partially displaced and the reporter is cleaved. The free reporter fluoresces

**Fig. 2.1. In TaqMan assays, hydrolysis probes are hydrolyzed by Taq polymerase.**

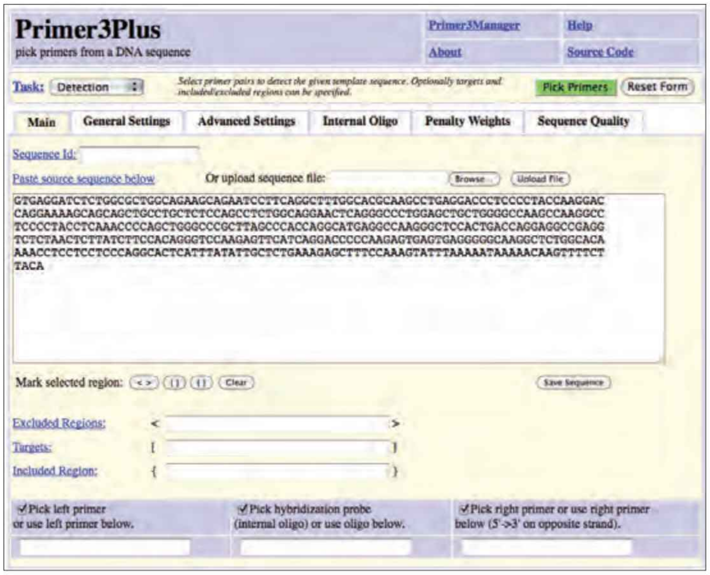
When designing probes, use the following guidelines:

- The probe sequence must be chosen between the two primers of the amplicon. Primer sequences cannot overlap with the probe, though they can sit directly next to one another
- The $T_m$ of a hydrolysis probe should be 3–10°C higher than that of the primers
- Choose a sequence within the target that has a GC content of 30–80%, and design the probe to anneal to the strand that has more Gs than Cs (so the probe contains more Cs than Gs)
- The probe should be <30 nucleotides long because distance between fluorophore and quencher affects baseline signal intensity. Longer probes or $T_m$ enhancers are recommended if needed to achieve the required $T_m$
- Probes should not have a G at the 5' end because this quenches the fluorescence signal even after hydrolysis
- Black Hole Quencher or other nonfluorescent quenchers are recommended
- $T_m$ enhancers for probes are recommended for single nucleotide polymorphism (SNP) and rare mutation detection assays in order to keep the background fluorescence to a minimum. Shorter probes discriminate better between single base differences in the target amplicon(s)

The QX100 and QX200 Systems are compatible with FAM and HEX or VIC dyes as well as duplex analysis of FAM/HEX or FAM/VIC. Using dye combinations in duplex assays enables a multiplex experiment and the ability to quantify and/or detect up to two targets per sample.

## Designing an Assay

Because manual balancing of all the constraints for primer and probe designs is challenging, time consuming, and error prone, we recommend software tools to ensure that all design constraints are satisfactorily followed. One widely used tool for PCR assay design is Primer3 (Whitehead Institute for Biomedical Research, Massachusetts Institute of Technology [MIT]). Building on this software, the easy-to-use Primer3Plus web interface (Untergasser et al. 2007) makes the process of assay design even easier (Figure 2.2).


Primer3Plus
pick primers from a DNA sequence
Primer3Manager
Help
About
Source Code
Pick Primers
Reset Form
Main
General Settings
Advanced Settings
Internal Oligo
Penalty Weights
Sequence Quality
Sequence Id:
Paste source sequence below
Or upload sequence file:
Browse...
Upload File
Mark selected region:
< >
[ ]
{ }
Clear
Save Sequence
Excluded Regions:
Targets:
Included Region:

Task: Detection

*Select primer pairs to detect the given template sequence. Optionally targets and included/excluded regions can be specified.*

```
GTGAGGATCTCTGGCGCTGGCAGAAGCAGAATCCTTCAGGCTTTGGCACGCAAGCCTGAGGACCCTCCCCTACCAAGGAC
CAGGAAAAGCAGCAGCTGCCTGCTCTCCAGCCTCTGGCAGGAACTCAGGGCCCTGGAGCTGCTGGGGCCAAGCCAAGGCC
TCCCCTACCTCAAACCCCAGCTGGGCCCGCTTAGCCCACCAGGCATGAGGCCAAGGGCTCCACTGACCAGGAGGCCGAGG
TCTCTAACTCTTATCTTCCACAGGGTCCAAGAGTTCATCAGGACCCCCAAGAGTGAGTGAGGGGGCAAGGCTCTGGCACA
AAACCTCCTCCTCCCAGGCACTCATTTATATTGCTCTGAAAGAGCTTTCCAAAGTATTTAAAAATAAAAACAAGTTTTCT
TACA
```

☑ Pick left primer or use left primer below.

☑ Pick hybridization probe (internal oligo) or use oligo below.

☑ Pick right primer or use right primer below (5'->3' on opposite strand).

Fig. 2.2. Main tab in Primer3Plus.

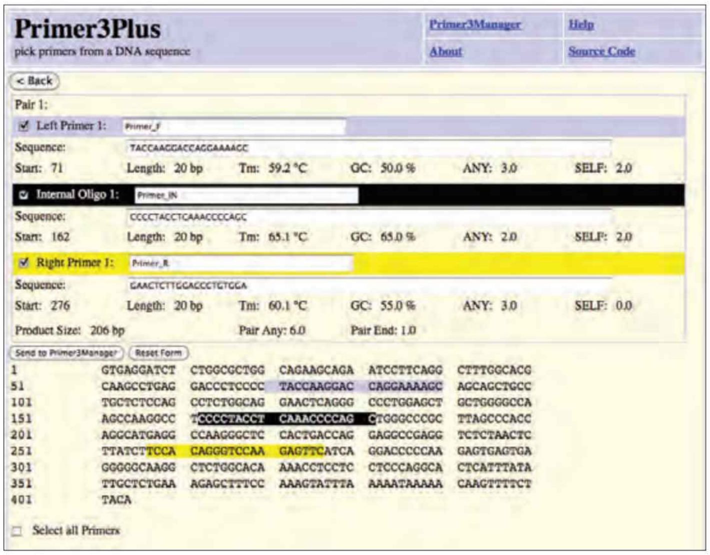
In the Main window, paste your target DNA sequence in the “Paste source sequence below” field.

We recommend the following changes to the default settings when designing ddPCR assays:

- In the General Settings window, change “Concentration of divalent cations” to 3.8, “Concentration of dNTPs” to 0.8, and “Mispriming/Repeat Library” to the correct organism
- In the Advanced Settings window, change both the “Table of thermodynamic parameters” and “Salt correction formula” to SantaLucia 1998
- In the Internal Oligo window, we recommend setting 15 for the minimum number of bases for the oligo. We recommend 64°C as the minimum $T_m$ for the probe, 65°C as the optimal $T_m$ for the probe, and 70°C as the maximum $T_m$ for the probe. These parameters can be relaxed to allow for smaller/larger oligos, which may be necessary for high GC or low GC targets. Oligo size should be no smaller than 13 and no larger than 30 nucleotides

**Note:** After you have made the desired changes in Primer3Plus, select **Save Settings** under General Settings and save these parameters in a file. To apply these settings in the future, upload them by selecting **Browse** in the General Settings tab, find this file, and click **Activate Settings**.

After you paste your target sequence into the Main window, click **Pick Primers** (Figure 2.3). The software provides one or more primer pairs to select or provides an explanation for why the software failed to arrive at any primers.



Pair 1:

☑ Left Primer 1: Primer_F
Sequence: TACCAAGGACCAGGAAAAGC
Start: 71 Length: 20 bp Tm: 59.2 °C GC: 50.0 % ANY: 3.0 SELF: 2.0

☑ Internal Oligo 1: Primer_IN
Sequence: CCCCTACCTCAAACCCCAGC
Start: 162 Length: 20 bp Tm: 65.1 °C GC: 65.0 % ANY: 2.0 SELF: 2.0

☑ Right Primer 1: Primer_R
Sequence: GAACTCTTGGACCCTGTGGA
Start: 276 Length: 20 bp Tm: 60.1 °C GC: 55.0 % ANY: 3.0 SELF: 0.0

Product Size: 206 bp Pair Any: 6.0 Pair End: 1.0

```
1           GTGAGGATCT  CTGGCGCTGG  CAGAAGCAGA  ATCCTTCAGG  CTTTGGCACG
51          CAAGCCTGAG  GACCCTCCCC  TACCAAGGAC  CAGGAAAAGC  AGCAGCTGCC
101         TGCTCTCCAG  CCTCTGGCAG  GAACTCAGGG  CCCTGGAGCT  GCTGGGGCCA
151         AGCCAAGGCC  TCCCCTACCT  CAAACCCCAG  CTGGGCCCGC  TTAGCCCACC
201         AGGCATGAGG  CCAAGGGCTC  CACTGACCAG  GAGGCCGAGG  TCTCTAACTC
251         TTATCTTCCA  CAGGGTCCAA  GAGTTCATCA  GGACCCCCAA  GAGTGAGTGA
301         GGGGGCAAGG  CTCTGGCACA  AAACCTCCTC  CTCCCAGGCA  CTCATTTATA
351         TTGCTCTGAA  AGAGCTTTCC  AAAGTATTTA  AAAATAAAAA  CAAGTTTTCT
401         TACA
```

Fig. 2.3. Assay design output.

Several important design features are not addressed by Primer3Plus (or the Primer3 MIT site).

- To ensure primer specificity, use tools such as BLAST (Basic Local Alignment Search Tool), hosted at the National Center for Biotechnology Information (NCBI), either in the “traditional” general search form (**www.ncbi.nlm.nih.gov/BLAST**) or a form tailored specifically to check that PCR primers (**http://www.ncbi.nlm.nih.gov/tools/primer-blast/**) match only your intended target

- Check that common SNPs do not land in your primer sequences

- Check for the secondary structure of the amplicon using the Mfold program (**http://mfold.rna.albany.edu/?q=mfold**). Primer binding sites should be predicted to be “open” (that is, not base-paired in a secondary structure) at the PCR annealing temperature

## Sample Preparation

The quality of the nucleic acid preparation from the sample of interest can impact ddPCR results. An optimized protocol should be used to extract the DNA or RNA from the raw material you are testing. Ensure that the sample has not been degraded, for example, by heating above 60°C. Although some PCR inhibitors are less detrimental to quantification accuracy in ddPCR than in other technologies, we recommend removing as many of these as possible during the nucleic acid purification phase. If known inhibitors cannot be readily removed, consider reducing their impact on the PCR reaction by diluting the sample 1:10.

For best results, restriction digestion of your DNA sample outside of the amplicon region is recommended. We have extensively investigated the use of endonucleases for fragmentation and found that a wide range of enzymes with 4-base and 6-base recognition sites perform satisfactorily for this purpose. The benefits of predigestion can be achieved with a wide range of enzyme concentrations. Considerations should be taken into account in the choice of enzyme for a particular locus:

1. The enzyme should not cut within the PCR target sequence itself.
2. It is best to use an enzyme that is insensitive to methylation to avoid incomplete fragmentation due to methylation of the target DNA.
3. In some instances, it is best to digest the target copy to the smallest size fragment that fully contains the amplicon footprint — preferably less than a few hundred base pairs.
4. If added at a relatively high concentration, some restriction enzyme buffers can result in a significant change in salt concentrations of the reaction mix. To avoid this, always digest in the lowest possible volume and mix with water before adding the digested DNA to the reaction mix.

## Adding DNA to the Reaction Mix

The recommended dynamic range of the QX100 or QX200 System is from 1 to 120,000 copies/20 µl reaction. There are about 120,000 copies in 400 ng of human DNA, assuming 1 copy/haploid genome. To estimate the number of copies/ng of DNA for your organism you must know the mass or the number of base pairs in the genome (see formula below). If the experiment entails quantifying samples known to have extremely high amounts of target molecules (such as next-generation sequencing [NGS] libraries), plan to reduce the starting sample accordingly. If the target copy number/genome is unknown, we recommend that you determine the optimal starting amount by doing four tenfold dilution series of each sample at the expected digital range. By assaying the four data points above and below the expected digital range, you ensure that one of the data points is within the optimal digital range.

To help determine copy number per genome, collect the following information:

1. If the source or species of the gDNA is known but the genome size of the organism of interest is unknown, refer to **http://www.cbs.dtu.dk/databases/DOGS/index.html** to determine the size of the genome in question.
2. Once the size of the genome is known, determine the mass of the genome using the following formula:

   $m = (n)\ (1.096 \times 10^{-21}\ \text{g/bp})$

   where $m$ is the genome mass in grams, and $n$ is the genome size in base pairs.

The following example calculates the mass of the human genome using the Celera Genomics estimate of

$3.0 \times 10^{9}$ bp (haploid):

$m = (3.0 \times 10^{9}\ \text{bp})\ (1.096 \times 10^{-21}\ \text{g/bp})$

$m = 3.3 \times 10^{-12}$ g or 3.3 pg

The example is relevant to any gene that is present at the normal rate of 2 copies/diploid genome, such as *RPP30*, and provides a basis for a digital screening experiment to determine the optimal digital range.

For sample DNA loading, follow these guidelines:

- Assess input DNA/RNA concentration using $A_{260}$ spectroscopy to ensure the target DNA/RNA concentration is being loaded within the dynamic range of detection

**Note:** An $A_{260}$ measurement depends on various factors and does not distinguish between intact and fragmented targets. As such it provides only an estimate of your DNA concentration.

- Add no more than 1 µg of digested DNA to the 20 µl reaction (final concentration of 50 ng/µl)
- Intact DNA requires restriction digestion for optimal performance, especially at a concentration above 3 ng/µl (60 ng/20 µl reaction). For copy number and absolute quantification, always do restriction digestion at all DNA concentrations unless you want to access proximal replicate sequences
- Do not perform a restriction digestion of the DNA sample within the amplicon sequence
- Fragmented DNA (for example, formalin-fixed, paraffin-embedded [FFPE] samples) may not need restriction digestion. However, some assays and/or targets require digestion for optimal target detection regardless of fragmentation
- cDNA does not require restriction digestion

# ddPCR Experimental Workflow

## Droplet Generation

For droplet generation, transfer 20 µl of a PCR reaction containing sample nucleic acid, primers (and probes for a TaqMan experiment), and the appropriate Bio-Rad ddPCR supermix to the middle rows of a DG8™ Cartridge for a QX100 or QX200 Droplet Generator (Figure 2.4). We recommend creating an initial reaction pool that is slightly more than 20 µl (22–25 µl) to ensure that 20 µl of mixture is transferred to the DG8 Cartridge. Reaction mixes should be combined and well mixed in a separate tube and not in the droplet generator cartridge. Reaction mixes should then be transferred to the DG8 Cartridge already preloaded in the DG8 Cartridge Holder.

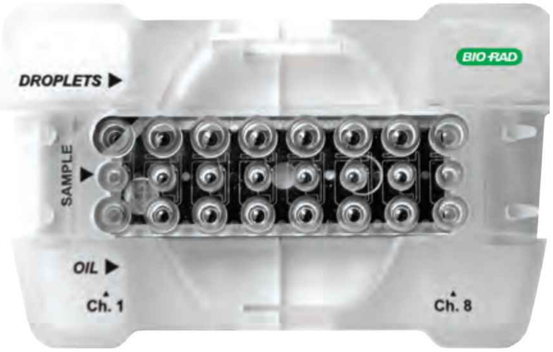


**Fig. 2.4. Loaded DG8 Cartridge.**

**Note:** Each DG8 Cartridge generates eight wells of droplets. Any unused wells on the cartridge must be filled with 1x ddPCR buffer control.

The Bio-Rad ddPCR supermixes have been formulated specifically to work with the droplet chemistry. Altering the components used in the QX200 Droplet Generator or using a different supermix will negatively impact results. A 1x final concentration of supermix must be used for proper droplet formation and proper target quantification.

After loading a 20 µl PCR reaction, load 70 µl of Droplet Generation Oil into the bottom wells of the DG8 Cartridge (Figure 2.4). Attach a gasket across the top of the DG8 Cartridge and place it into the QX200 Droplet Generator (Figure 2.5). The Droplet Generator produces about 20,000 droplets per sample in about 2.5 min for eight samples. Droplets should be transferred to a 96-well PCR plate by pipetting gently.

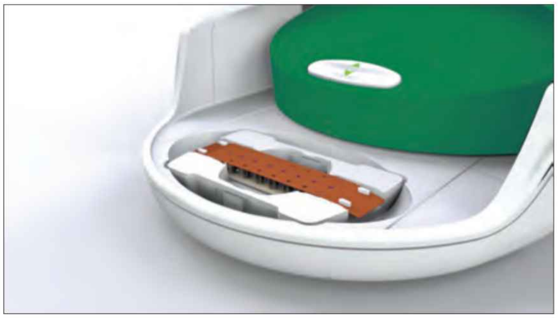


**Fig. 2.5. Loaded DG8 Cartridge placed in the QX200 Droplet Generator.**

We recommend designing your experimental plate layout on a 96-well plate in columns because the cartridge contains eight wells.

## PCR

After generating droplets in the DG8 Cartridge, pipet the droplets from the top wells of the cartridge into a PCR plate. The PCR plate should be heat sealed using Bio-Rad's PX1™ PCR Plate Sealer and pierceable foil heat seal.

**Note:** Using an alternative seal with glue can damage the Droplet Reader.

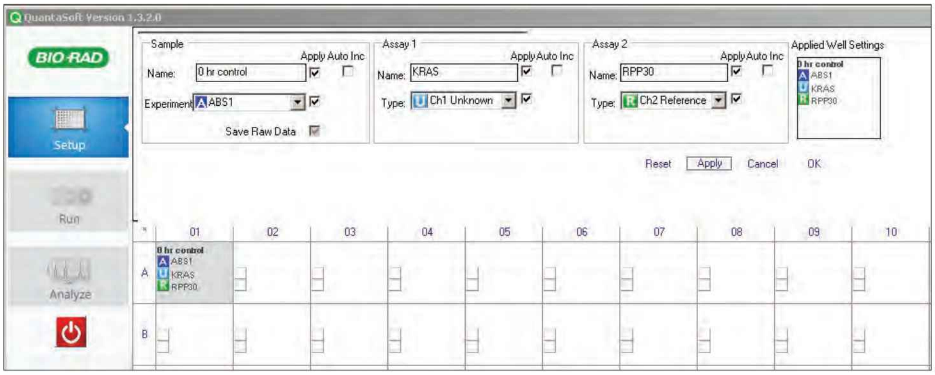
After heat sealing, place the PCR plate in a thermal cycler for PCR using the following guidelines.

- Use a recommended thermal cycling protocol
- Use a 2.5°C/sec ramp rate to ensure each droplet reaches the correct temperature for each step during the cycling
- 40 cycles of PCR is enough for an optimized ddPCR assay. Do not exceed 50 cycles
- After PCR, the plate can be left in the thermal cycler overnight at 10°C or stored at 4°C. Do not store the plate for more than 3–4 days before running it in a QX100 or QX200 Droplet Reader

## Setting Up an Experiment in QuantaSoft™ Software

From the computer attached to the droplet reader, open QuantaSoft Software in the setup mode and design a new plate with a layout according to your experimental design. Detailed instructions for how to set up a new experiment and interpret ddPCR data can be found in the user manual.

Double click on a well in the plate layout to open the Well Editor dialog box. Designate the sample name, experiment type, and which assays correspond to which channels, such as FAM and HEX (Figure 2.6). You can select several contiguous wells at one time using shift + double click or select non-contiguous wells using Ctrl + double click. Either selection will bring up the labeling menu. In the Well Editor dialog box, input sample names and use the dropdown menu to designate the experiment type.


QuantaSoft Version 1.3.2.0

Sample
Name: 0 hr control ☑ ☐
Experiment: A ABS1 ☑
Save Raw Data ☑

Assay 1
Name: KRAS ☑ ☐
Type: U Ch1 Unknown ☑

Assay 2
Name: RPP30 ☑ ☐
Type: R Ch2 Reference ☑

Applied Well Settings
0 hr control
A ABS1
U KRAS
R RPP30

A01: 0 hr control, A ABS1, U KRAS, R RPP30

Fig. 2.6. Labeling the plate.

There are three types of experiments that can be selected for each well:

- ABS — absolute quantification
- RED — rare target sequence detection (rare event detection)
- CNV — copy number variation to measure the concentration of target relative to the concentration of a reference

Select **Apply** to load the wells and when finished select **OK**. Once the plate layout is complete, select **Run** to begin the droplet reading process.

## Droplet Reading

Following PCR amplification of the nucleic acid target in the droplets, place the PCR plate in a QX100 or QX200 Droplet Reader. The Droplet Reader and QuantaSoft Software count the PCR-positive and PCR-negative droplets to provide absolute quantification of target DNA.

Droplet reading considerations are as follows:

- Before a run, the instrument can be set to interrogate droplets either in rows or columns
- Ensure there is enough Droplet Reader Oil in the instrument and the waste is empty before a run
- Each sample is processed individually and interrogated for both FAM and HEX (or VIC) fluorescence
- Data from 12,000–16,000 droplets are used in concentration calculations
- The reader measures fluorescence intensity of each droplet and detects the size and shape as droplets pass the detector; droplets are excluded if they do not meet quality metrics

## Data Analysis Using QuantaSoft Software

After the QX100 or QX200 Droplet Reader has finished interrogating all wells, use QuantaSoft Software to analyze the data in each well. It automatically opens the first well with data to begin the analysis. If the plate was set up for ABS analysis, automatic thresholding determines concentrations and populates the data tables in the analysis mode of the software.

**Important:** The concentration reported is copies/µl of the final 1x ddPCR reaction.

Well data must meet certain quality metrics before QuantSoft Software will automatically calculate a threshold above which droplets are considered positive. The threshold may be manually adjusted on a well-by-well basis or across an entire plate (Figure 2.7).

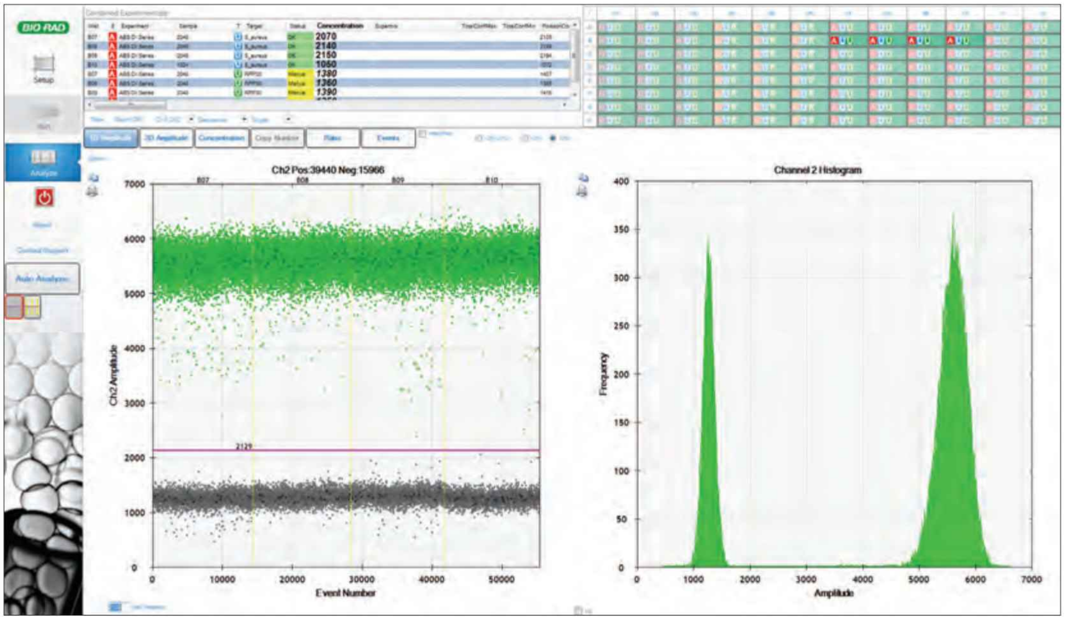

BIO-RAD
Setup
Analyze
Auto Analyze
Concentration
2070
2140
2150
1050
1380
1360
1390
2D Amplitude
Concentration
Copy Number
Ratio
Events
Ch2 Pos:39440 Neg:15966
B07
B08
B09
B10
7000
6000
5000
4000
3000
2000
1000
0
Ch2 Amplitude
2129
0
10000
20000
30000
40000
50000
Event Number
Channel 2 Histogram
400
350
300
250
200
150
100
50
0
Frequency
0
1000
2000
3000
4000
5000
6000
7000
Amplitude

Fig. 2.7. Setting thresholds in a 1-D plot.

**Note:** Use 1-D plots for temperature gradient experiments and wells containing single assays. For all experiments involving duplex assays, set thresholds in the 2-D plot view (Figure 2.8).

For correct quantification of a single-color experiment, use the thresholding tools to ensure correct designation of the populations as negatives (gray) and positives (blue or green). The thresholding tools are on the left side of the screen. There is an autoanalysis tool as well as two manual thresholding tools — one for individual wells and one for all selected wells. Once you have chosen the desired tool, place the crosshair cursor over the place where you would like to set the threshold and click on the chart. The droplets will change color when you are done.

To analyze the data for a multiplex experiment, select **Analyze** and click the **2-D Amplitude** tab.

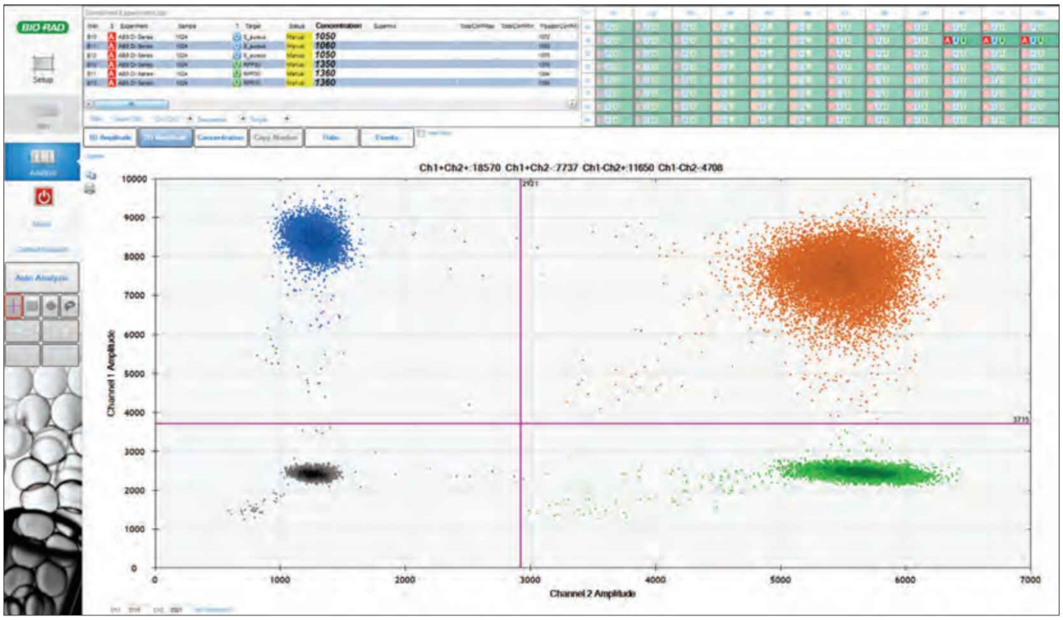

Ch1+Ch2+:18570 Ch1+Ch2-:7737 Ch1-Ch2+:11650 Ch1-Ch2-:4708
Channel 1 Amplitude
Channel 2 Amplitude
10000
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
0
1000
2000
3000
4000
5000
6000
7000
2921
3715
Concentration
1050
1060
1050
1350
1360
1360

Fig. 2.8. Setting thresholds in a 2-D plot.

For correct quantification of a duplex experiment, use the thresholding tools to ensure correct designation of the droplet populations as double negative (gray), FAM positive (blue), VIC/HEX positive (green), and double positive (brown = positive for FAM and HEX/VIC in the same droplet). There is an autoanalysis tool as well as several different manual thresholding tools including a "free-draw" tool. Once you have selected the desired color tab and draw tool, click on the chart and select the droplets. They will change color when you are done.

## Merging Wells

Selecting the merged button in QuantaSoft Software combines the positive and negative droplets from all the wells with the same name and analyzes these data together as a single experiment. You can display merged and single wells simultaneously or individually. All merged-well data are displayed with a closed symbol (Figure 2.9). In contrast, single-well data are displayed with an open symbol (see Figure 1.11).

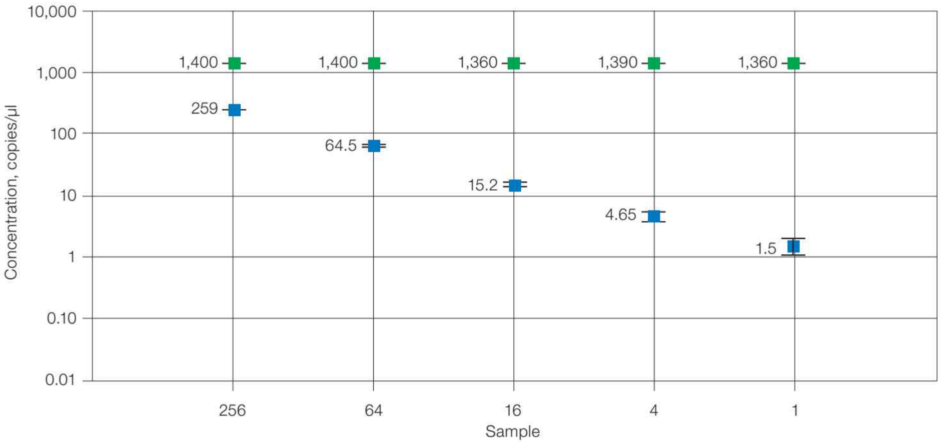


Fig. 2.9. Merged well data.

# QuantaSoft Analysis Pro Software

The Bio-Rad QuantaSoft Analysis Pro (QuantaSoft AP) Software is a complementary analysis tool that enables data analysis of multiplex experiments and advanced applications and assay strategies for Droplet Digital PCR (ddPCR). QuantaSoft AP is for use with data files generated on the QX100 or QX200 Droplet Reader using QuantaSoft Standard Edition Software versions 1.4 and later.

Benefits of using QuantaSoft AP include:

- Analyze multiple targets within a well using amplitude multiplexing and probe mixing strategies
- Detect genome edit and non-wild type events with a “drop-off assay” analysis option
- Visualize and export data with improved flexibility
- Customize an experiment using an advanced droplet classification method for cutting edge research with ddPCR technology

## QuantaSoft Analysis Pro Installation

QuantaSoft Analysis Pro (AP) software is available to download and install from **www.bio-rad.com**. There is no installation disc provided for the software. QuantaSoft Standard Edition is still required to operate the QX100 or QX200 Droplet Reader and collect data

**Table 2.1. Minimum system requirements.**

| System | Minimum |
|---|---|
| CPU | 17 Quad Core |
| Processor speed | 2.40 GHz |
| Installed RAM | 8 GB RAM |
| Hard drive | 500 GB, 7,200 rpm |
| Video graphics | 1,696 MB |
| Dedicated | 64 MB |
| Operating system | Windows 7 or Windows 10, 64 bit |

QuantaSoft Analysis Pro will only run at a resolution of 1,920 x 1,080 and 100% font.

## Installation

1. Go to **http://www.bio-rad.com/en-us/product/qx200-droplet-digital-pcr-system** and click on the Download tab. Download QQuantasoftAP_1.0.596_Setup.exe (QuantaSoft Analysis Pro Installer).
2. From your download folder, double click the file to begin installation.
3. The QuantaSoft Analysis Pro InstallShield® Wizard will pop up and guide you through the installation process.
4. Follow the onscreen instructions and click Finish to complete installation.
5. To access the End-User License Agreement (EULA), open QuantaSoft AP, click the Help icon, and click the EULA link.

**Note:** Optional: If Microsoft Visual C++ 2015 Update 1 Redistribution Package (X64) has not been installed on your computer yet, you will be prompted to install it. Optional: If .NET Framework 4.5.2 has not been installed on your computer yet, you will be prompted to install it.

## Using QuantaSoft Analysis Pro

QuantaSoft™ Analysis Pro (AP) software opens and analyzes files generated by QuantaSoft Software v1.4 and later. This software is for analysis only; it cannot be used to run the QX100 or QX200 Droplet Reader. Additionally, QuantaSoft AP cannot open .qlb, .qlt, or any files from QuantaSoft v1.7 Regulatory Edition.

QuantaSoft AP can open the following files:

**Results (*.qlp)** — user-defined plate information and collected data from a run completed on the QX100 or QX200 Droplet Reader

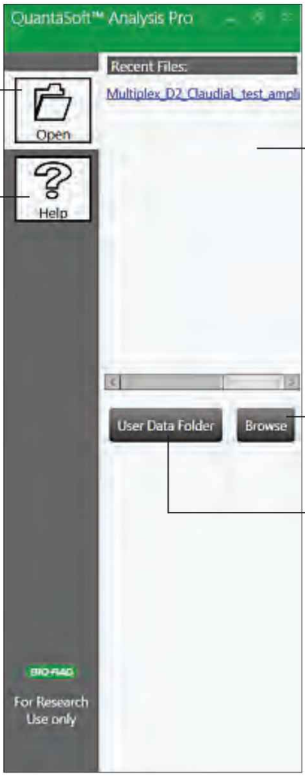
**Results (*.ddpcr)** — user files that were created by QuantaSoft AP

**Plate Setup File (*.ddplt)** — plate setup file that is created in QuantaSoft AP and can be saved for expedited setup of additional plates

## How to Open a File

### Opening QuantaSoft AP and Files

1. QuantaSoft AP can be opened by double clicking the shortcut on the desktop or through the start menu.
2. QuantaSoft AP can open .qlp files generated in QuantaSoft v 1.4 or later, .ddpcr and .ddplt files.
    a. .ddPCR files can be opened by double clicking a file or by dragging and dropping it into QuantaSoft Analysis Pro window.
    b. .ddplt files can be opened by clicking on Plate Editor Tools in the menu bar and selecting Import Plate Setup.
    c. .qlp files can be opened by dragging and dropping into the analysis pro window or by clicking the **Browse** to find and open the file.


QuantaSoft™ Analysis Pro
Recent Files:
Multiplex_D2_Claudial_test_ampli
Open
Help
User Data Folder
Browse
BIO-RAD
For Research Use only

The Open Files Tab, open .qlp and .ddPCR files from this screen.

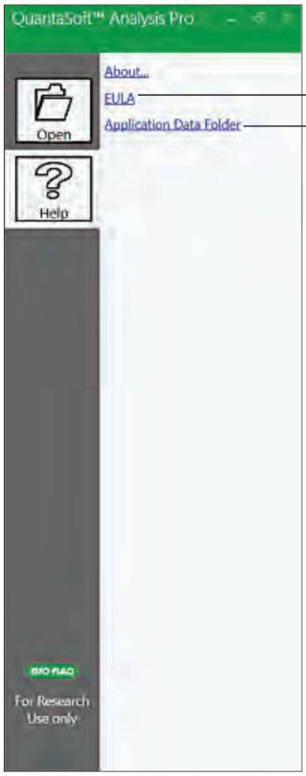
Help Tab

Links to 10 files opened most recently, listed from newest to oldest. To open .qlp files, drag and drop them here.

Choose file from computer; opens the last folder from which a file was opened.

Click to access DATA folder in QuantaSoft AP main folder.

Fig. 2.10. Control panel of QuantaSoft Analysis Pro, Open Files Tab.


QuantaSoft™ Analysis Pro
About...
EULA
Application Data Folder
Open
Help
BIO-RAD
For Research Use only

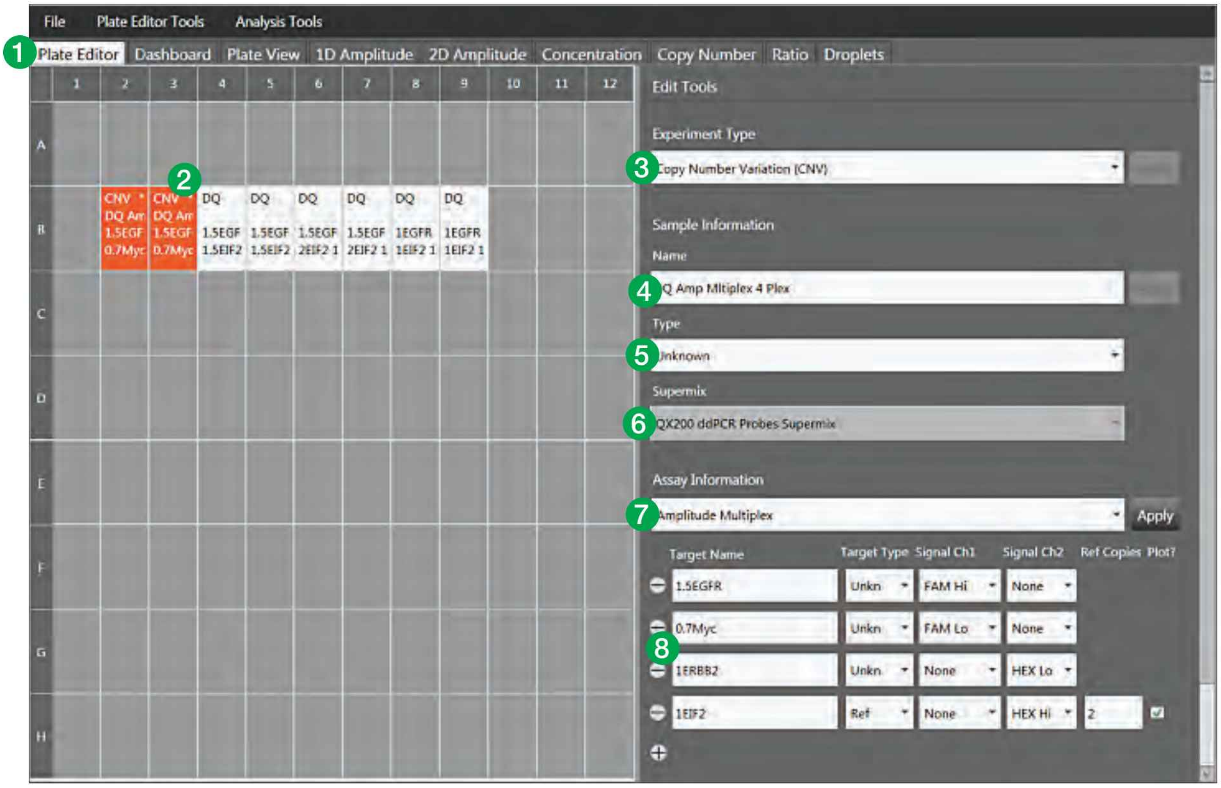
Click to access EULA.

Directs you to LOGS folder. Zip entire folder and send to tech support for troubleshooting.

Fig. 2.11. Control panel of QuantaSoft Analysis Pro, Help Tab.

## Overview of Screens and Buttons

Results files will open to the Dashboard tab. You can navigate to any of the software analysis tabs, which are discussed in detail in this section.



| | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |
|---|---|---|---|---|---|---|---|---|
| B | CNV *<br>DQ Am<br>1.5EGF<br>0.7Myc | CNV *<br>DQ Am<br>1.5EGF<br>0.7Myc | DQ<br>1.5EGF<br>1.5EIF2 | DQ<br>1.5EGF<br>1.5EIF2 | DQ<br>1.5EGF<br>2EIF2 1 | DQ<br>1.5EGF<br>2EIF2 1 | DQ<br>1EGFR<br>1EIF2 1 | DQ<br>1EGFR<br>1EIF2 1 |

Experiment Type: Copy Number Variation (CNV)

Sample Information

Name: Q Amp Mltiplex 4 Plex

Type: Unknown

Supermix: QX200 ddPCR Probes Supermix

Assay Information: Amplitude Multiplex

| Target Name | Target Type | Signal Ch1 | Signal Ch2 | Ref Copies | Plot? |
|---|---|---|---|---|---|
| 1.5EGFR | Unkn | FAM Hi | None | | |
| 0.7Myc | Unkn | FAM Lo | None | | |
| 1ERBB2 | Unkn | None | HEX Lo | | |
| 1EIF2 | Ref | None | HEX Hi | 2 | ☑ |

**Fig. 2.12. Plate Editor Tab** – In this screen you will configure information for each plate, selecting parameters that are required for all analysis modes. All information entered here is carried through each tab.

### Legend Overview

1. Click different tabs to access plots, tables and analyses for the plate.
2. Select an individual or multiple wells to lay out experiment.
3. **Experiment Type** — Select from dropdown list to pick experiment type.
4. **Name** — Type a name for the sample selected.
5. **Type** — Select sample type: unknown, NTC, positive control, or negative control.
6. The Supermix is selected during plate setup prior to running the QX100 or QX200 in QuantaSoft Software and cannot be changed in QuantaSoft Analysis Pro.
7. **Assay Information** — Select the type of assay being run: Simplex/Duplex, Amplitude Multiplex, Probe Mix Triplex, or Advanced Classification Mode.
8. Name your targets, select either unknown or reference, and identify dye for each detection channel. If Copy Number Variation is selected under Experiment Type you will have the option to input the Copy Number for the reference target.

**Note:** Plate information saved in the .qlp file will be automatically imported into the relevant Edit Tools fields.

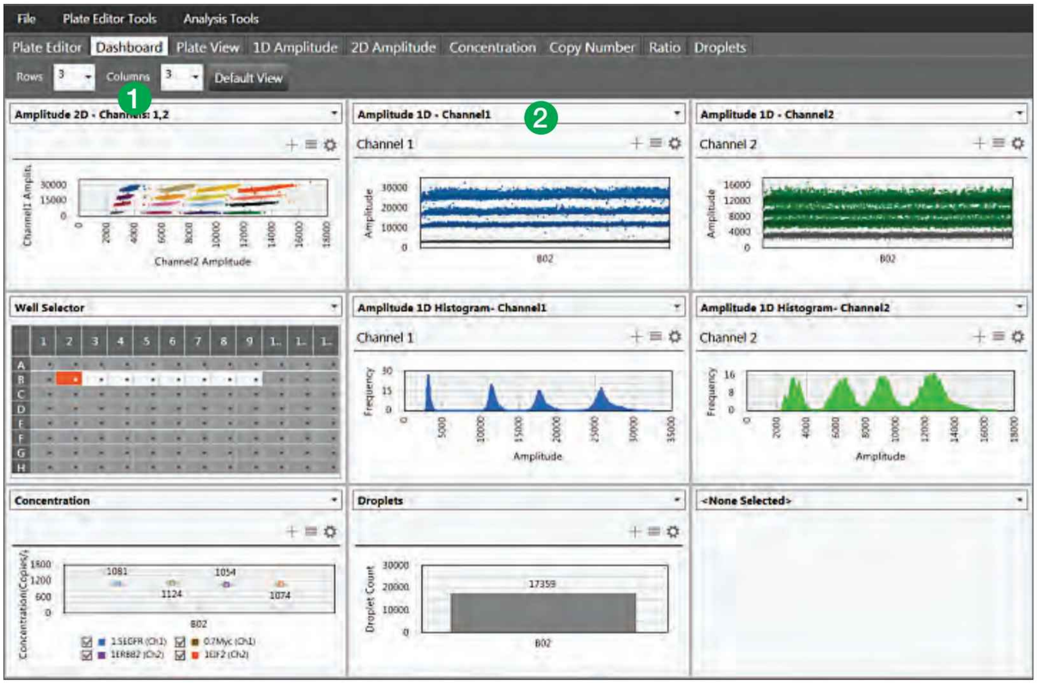

File
Plate Editor Tools
Analysis Tools
Plate Editor
Dashboard
Plate View
1D Amplitude
2D Amplitude
Concentration
Copy Number
Ratio
Droplets
Rows
Columns
Default View
1
2
Amplitude 2D - Channels: 1,2
Channel1 Amplitude
Channel2 Amplitude
Amplitude 1D - Channel1
Channel 1
Amplitude 1D - Channel2
Channel 2
Amplitude
B02
Well Selector
Amplitude 1D Histogram- Channel1
Amplitude 1D Histogram- Channel2
Frequency
Concentration
Concentration(Copies/µL)
1081
1124
1054
1074
Droplets
Droplet Count
17359
<None Selected>

Fig. 2.13. Dashboard Tab.

## Legend Overview

A single screen view with a summary of all analyses. Within this tab you can select which experiment results should be summarized for the selected experiment type. Data on this screen will update with changes made in other analysis tabs.

1. Customize dashboard by determining how many windows you want displayed on the screen.
2. Use the drop down menu in each block to select which plots, tables, and options are displayed.

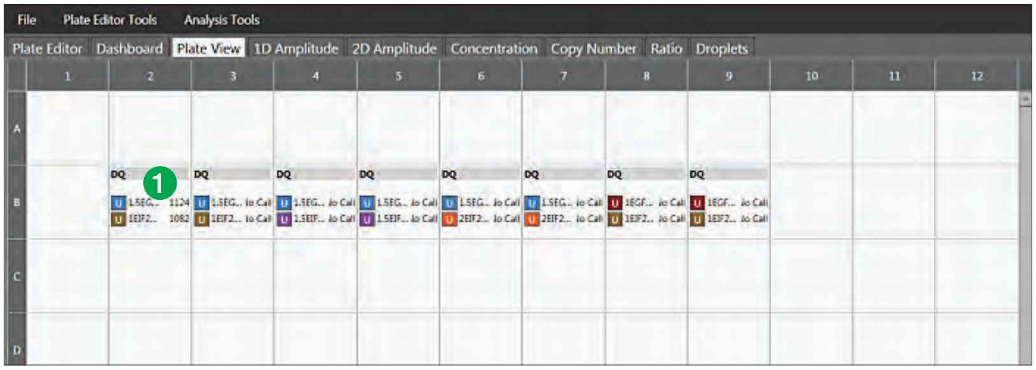
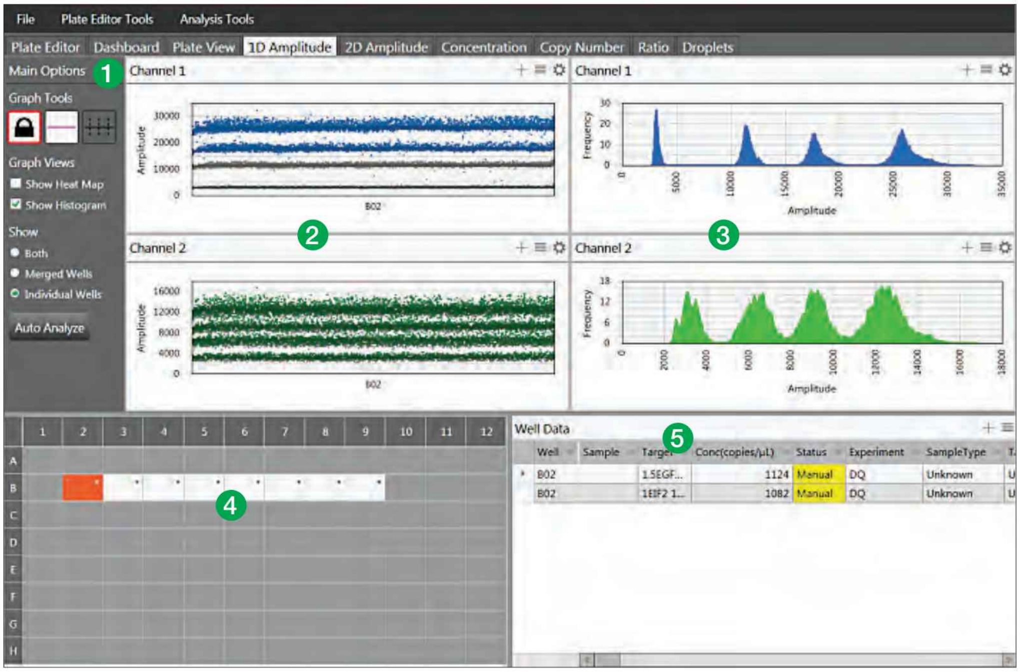

File
Plate Editor Tools
Analysis Tools
Plate Editor
Dashboard
Plate View
1D Amplitude
2D Amplitude
Concentration
Copy Number
Ratio
Droplets
1
2
3
4
5
6
7
8
9
10
11
12
A
B
C
D
DQ
1

Fig. 2.14. Plate View Tab.

## Legend Overview

A single screen view that shows results in a plate layout.

1. Identifies the experiment type, sample name, target name, target type, and concentration of each target by well in a plate layout.


File
Plate Editor Tools
Analysis Tools
Plate Editor
Dashboard
Plate View
1D Amplitude
2D Amplitude
Concentration
Copy Number
Ratio
Droplets
Main Options
1
Graph Tools
Graph Views
☐ Show Heat Map
☑ Show Histogram
Show
● Both
● Merged Wells
○ Individual Wells
Auto Analyze
Channel 1
Amplitude
30000
20000
10000
0
B02
Channel 1
Frequency
30
20
10
0
0
5000
10000
15000
20000
25000
30000
35000
Amplitude
2
3
Channel 2
Amplitude
16000
12000
8000
4000
0
B02
Channel 2
Frequency
18
12
6
0
0
2000
4000
6000
8000
10000
12000
14000
16000
18000
Amplitude
1
2
3
4
5
6
7
8
9
10
11
12
A
B
C
D
E
F
G
H
4
Well Data
5

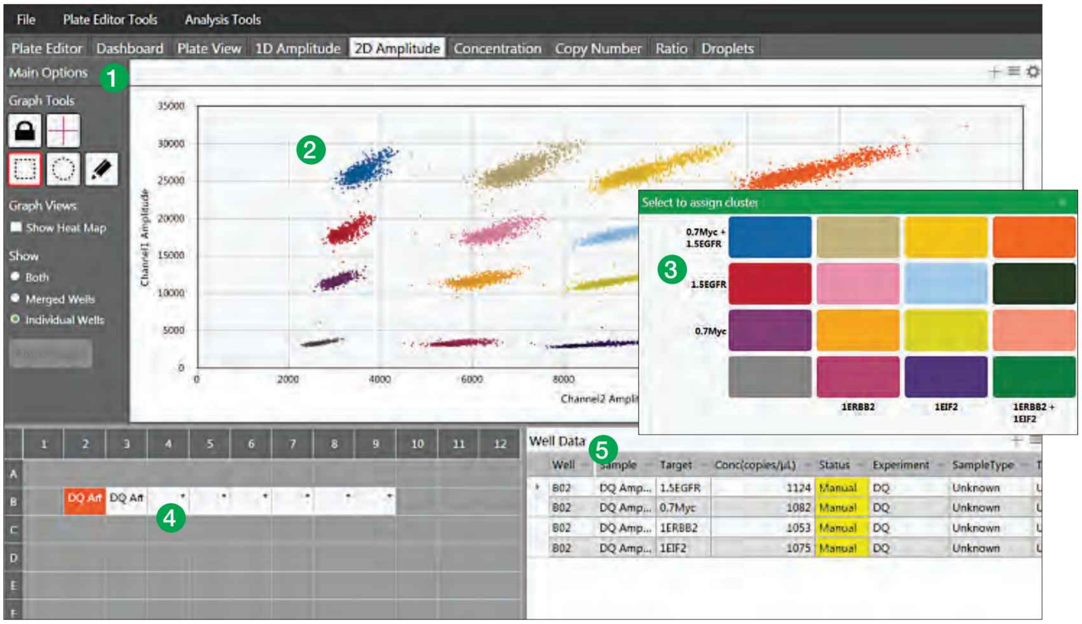
| Well | Sample | Target | Conc(copies/µL) | Status | Experiment | SampleType | T |
|---|---|---|---|---|---|---|---|
| B02 |  | 1.5EGF... | 1124 | Manual | DQ | Unknown | U |
| B02 |  | 1EIF2 1... | 1082 | Manual | DQ | Unknown | U |

**Fig. 2.15. 1D Amplitude Tab.**

## Legend Overview

A single screen to view 1D fluorescence amplitude plots and data results for the plate.

1. **Graph Tools**
   - **Threshold**
     - **View Mode** — prevents changing of thresholds
     - **Threshold Single Well** — establish threshold for positive and negative droplets in either Channel 1 or Channel 2 by individual well
     - **Threshold Multiple Wells** — establish threshold for positive and negative droplets in either Channel 1 or Channel 2 by multiple wells
   - **Graph Views** — toggle checkboxes to display or hide histograms and heat maps
   - **Show**
     - **Both** — all selected wells will be plotted for Merged and Individual Wells
     - **Merged Wells** — merges data from all selected wells into a single data point
     - **Individual Wells** — plots each well as a separate data point in any data plot
   - **Auto Analyze** — sets automatic thresholds in the well or group of wells selected, overriding any previously set thresholds. Auto analysis is only available for simplex/duplex experiments; the button will not be active in other analysis modes.
2. **1-D Fluorescence Amplitude Plots** — shows all accepted droplets for a selected well or wells on the plate in both Channel 1 (FAM/EvaGreen®) and Channel 2 (HEX/VIC). Once thresholds are established, negative droplets are displayed in grey, while positive droplets in Channel 1 are blue and positive droplets in Channel 2 are green.
3. **1-D Fluorescence Amplitude Histograms** — displays frequency of droplets at specific fluorescence amplitudes for each well or group of wells selected. The Channel 1 (FAM/EvaGreen®) histogram is shown in blue and Channel 2 (HEX/VIC) histogram in green.
4. **Well selector** — click on one well or select multiple wells to analyze in this tab.
5. **Data Table** — results for a selected well or group of wells, displayed by well number and sortable by any column by clicking on its header.


File
Plate Editor Tools
Analysis Tools
Plate Editor
Dashboard
Plate View
1D Amplitude
2D Amplitude
Concentration
Copy Number
Ratio
Droplets
Main Options
1
Graph Tools
Graph Views
☐ Show Heat Map
Show
● Both
● Merged Wells
○ Individual Wells
2
Channel1 Amplitude
35000
30000
25000
20000
15000
10000
5000
0
0
2000
4000
6000
8000
Channel2 Ampli
Select to assign cluster
3
0.7Myc + 1.5EGFR
1.5EGFR
0.7Myc
1ERBB2
1EIF2
1ERBB2 + 1EIF2
1
2
3
4
5
6
7
8
9
10
11
12
A
B
C
D
E
F
DQ Am
DQ Am
4
Well Data
5

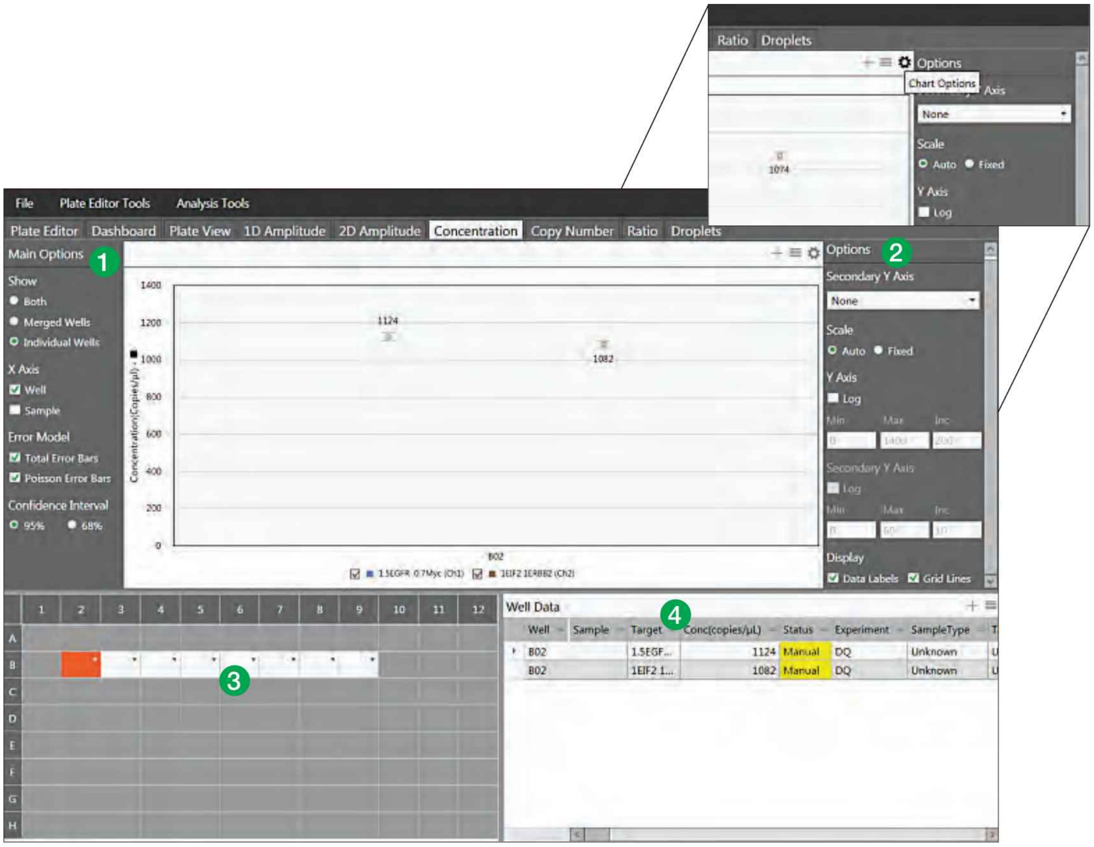
| Well | Sample | Target | Conc(copies/µL) | Status | Experiment | SampleType | T |
|---|---|---|---|---|---|---|---|
| B02 | DQ Amp... | 1.5EGFR | 1124 | Manual | DQ | Unknown | U |
| B02 | DQ Amp... | 0.7Myc | 1082 | Manual | DQ | Unknown | U |
| B02 | DQ Amp... | 1ERBB2 | 1053 | Manual | DQ | Unknown | U |
| B02 | DQ Amp... | 1EIF2 | 1075 | Manual | DQ | Unknown | U |

**Fig. 2.16. 2D Amplitude Tab.**

## Legend Overview

A single screen to view 2D fluorescence amplitude plots and data results for the plate.

1. **Graph Tools**
   - **Threshold**
     - **View Mode** — prevents changing of thresholds
     - **Line mode** — establishes thresholds across both Channel 1 and Channel 2, dividing droplets into distinct clusters separated by different colors. Click anywhere on the plot to apply "best fit" threshold lines to the data. Adjust the threshold values by dragging the lines horizontally or vertically in the plot area, by entering a new threshold value in the boxes at the end of each threshold line, or by clicking the up and down arrows to incrementally adjust the numbers.
     - **Cluster mode** — establishes thresholds across both Channel 1 and Channel 2, dividing droplets into distinct clusters separated by different colors. Click any of the three Threshold Cluster Mode buttons (square, circle or free form). Draw the shape chosen around the cluster of droplets to classify them.
   - **Graph Views** — toggle checkbox to display or hide heat map in amplitude events chart
   - **Show**
     - **Both** — all selected wells will be plotted for Merged and Individual Wells
     - **Merged Wells** — merges data from all selected wells into a single data point
     - **Individual Wells** — plots each well as a separate data point in any data plot
2. **2-D amplitude events chart** — graphical depiction of the amplitude of all qualified droplets per well or group of wells in channel 1 or channel 2.
3. **Cluster Threshold Mode (Pop-up, inset)** — when using the cluster mode tools to draw a shape around a cluster of droplets, the pop-up tool will appear to help you select the correct target combination when you release the mouse button. Target names are shown along the two axes based on the signal values assigned during plate setup. Click the colored box that best represents the location of the cluster of droplets selected to automatically classify the droplets as positive or negative for each given target.
4. **Well selector** — select one or multiple wells to analyze in this tab.
5. **Data Table** — results for a selected well or group of wells, displayed by well number and sortable by any column by clicking on its header.


Ratio
Droplets
Options
Chart Options
Axis
File
Plate Editor Tools
Analysis Tools
Plate Editor
Dashboard
Plate View
1D Amplitude
2D Amplitude
Concentration
Copy Number
Ratio
Droplets
1
2
3
4
1400
1200
1000
800
600
400
200
0
Concentration(Copies/µL)
1124
1082
1074
B02
1.5EGFR 0.7Myc (Ch1)
1EIF2 1ERBB2 (Ch2)
1
2
3
4
5
6
7
8
9
10
11
12
A
B
C
D
E
F
G
H

Main Options

Show
○ Both
● Merged Wells
○ Individual Wells

X Axis
☑ Well
☐ Sample

Error Model
☑ Total Error Bars
☑ Poisson Error Bars

Confidence Interval
○ 95% ● 68%

Options

Secondary Y Axis: None

Scale
○ Auto ● Fixed

Y Axis
☐ Log

| Min | Max | Inc |
|---|---|---|
| 0 | 1400 | 200 |

Secondary Y Axis
☐ Log

| Min | Max | Inc |
|---|---|---|
| 0 | 60 | 10 |

Display
☑ Data Labels ☑ Grid Lines

Well Data

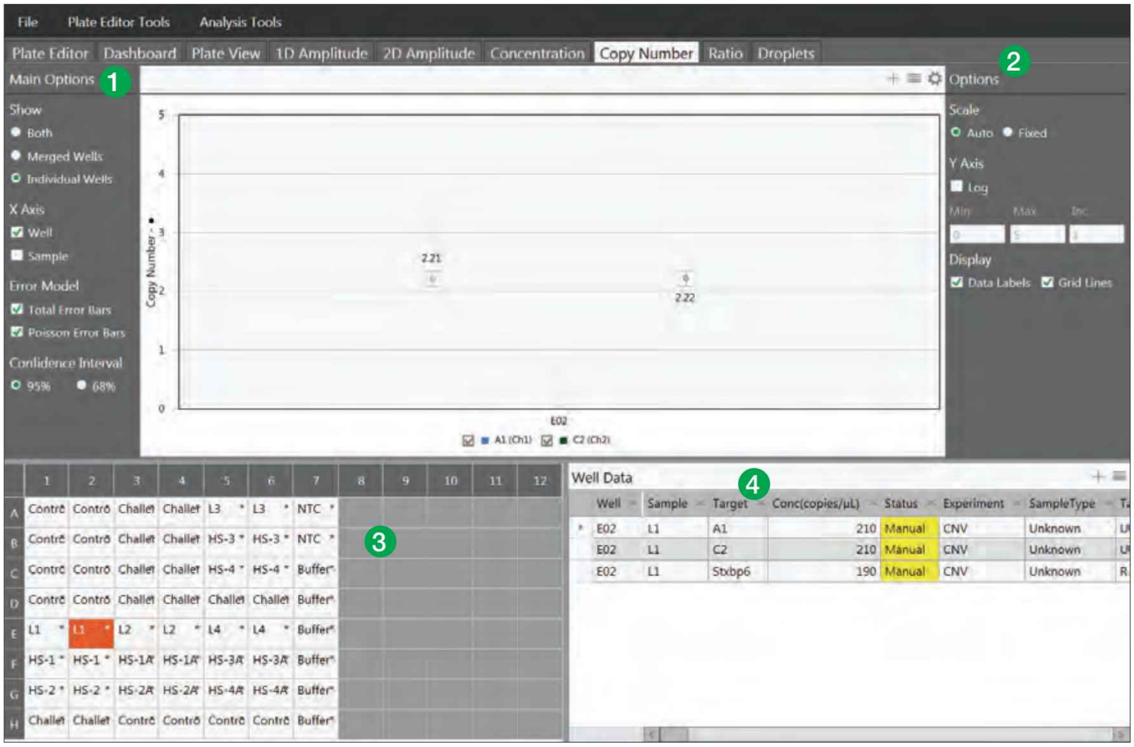
| Well | Sample | Target | Conc(copies/µL) | Status | Experiment | SampleType | T |
|---|---|---|---|---|---|---|---|
| B02 | | 1.5EGF... | 1124 | Manual | DQ | Unknown | U |
| B02 | | 1EIF2 1... | 1082 | Manual | DQ | Unknown | U |

**Fig. 2.17. Concentration tab.** Inset: To expand the options menu of any plot or chart, click on the gear icon at the top right of the chart. Click on the icon with three horizontal lines to bring up the chart menu, which is also available by right clicking on the plot area. The plot image can be saved, exported or printed from this menu. Click on the + icon to expand any plot area to full screen.

## Legend Overview

This tab contains the concentration plot for individual or multiple wells and allows you to adjust plot parameters.

1. **Main Options**
   - **Show**
     - Both — all selected wells, both merged and individual, will be plotted
     - Merged Wells — merges data from all selected wells into a single data point on the plot
     - Individual Wells — plots each well as a separate data point
   - **X Axis** — choose how the x axis labels are displayed and plotted, by sample name or well number
   - **Error Model** — select Total Error Bars, Poisson Error Bars, or both
   - **Confidence Interval** — select either 95% or 68%; 95% is the default
2. **Options** – click the gear icon to bring up chart options menu, see inset above.
   - **Secondary Y-Axis** — enable an optional secondary axis to plot channel 2 concentration, ratio, copy number, or fractional abundance
   - **Scale** — choose auto or fixed
   - **Log** — change the axis to a logarithmic scale
   - **Range** — set minimum, maximum, and increment for y-axis and secondary y-axis (if chosen)
   - **Display** — toggle checkbox to choose whether to display Data Labels and Grid Lines
3. **Well selector** — select one or multiple wells to analyze in this tab
4. **Data Table** — results for a selected well or group of wells, displayed by well number and sortable by any column by clicking on its header.


File
Plate Editor Tools
Analysis Tools
Plate Editor
Dashboard
Plate View
1D Amplitude
2D Amplitude
Concentration
Copy Number
Ratio
Droplets
Main Options
1
Show
● Both
● Merged Wells
○ Individual Wells
X Axis
☑ Well
☐ Sample
Error Model
☑ Total Error Bars
☑ Poisson Error Bars
Confidence Interval
○ 95%
● 68%
Copy Number
5
4
3
2
1
0
2.21
2.22
E02
☑ A1 (Ch1)
☑ C2 (Ch2)
2
Options
Scale
○ Auto
● Fixed
Y Axis
☐ Log
Min
Max
Inc
Display
☑ Data Labels
☑ Grid Lines
3
Well Data
4

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A | Contro | Contro | Challe | Challe | L3 | L3 | NTC | | | | | |
| B | Contro | Contro | Challe | Challe | HS-3 | HS-3 | NTC | | | | | |
| C | Contro | Contro | Challe | Challe | HS-4 | HS-4 | Buffer | | | | | |
| D | Contro | Contro | Challe | Challe | Challe | Challe | Buffer | | | | | |
| E | L1 | L1 | L2 | L2 | L4 | L4 | Buffer | | | | | |
| F | HS-1 | HS-1 | HS-1A | HS-1A | HS-3A | HS-3A | Buffer | | | | | |
| G | HS-2 | HS-2 | HS-2A | HS-2A | HS-4A | HS-4A | Buffer | | | | | |
| H | Challe | Challe | Contro | Contro | Contro | Contro | Buffer | | | | | |

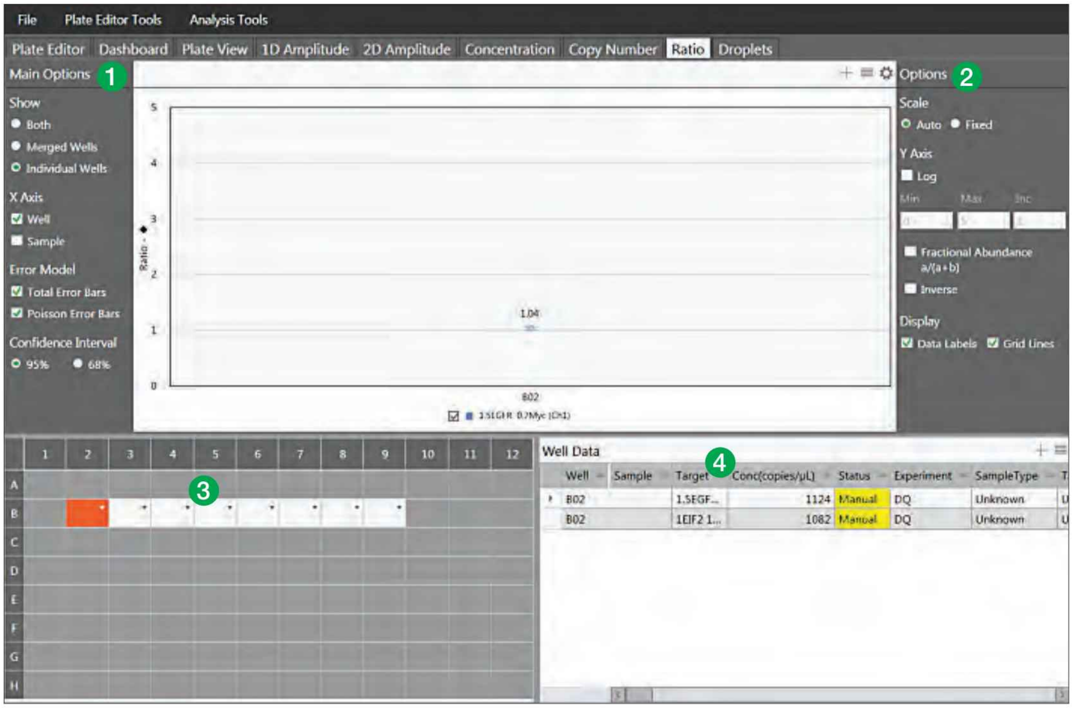
| Well | Sample | Target | Conc(copies/µL) | Status | Experiment | SampleType |
|---|---|---|---|---|---|---|
| E02 | L1 | A1 | 210 | Manual | CNV | Unknown |
| E02 | L1 | C2 | 210 | Manual | CNV | Unknown |
| E02 | L1 | Stxbp6 | 190 | Manual | CNV | Unknown |

Fig. 2.18. Copy Number Tab.

## Legend Overview

This tab shows the copy number plot for individual or multiple wells and allows you to adjust plot parameters.

1. **Main Options**
   - **Show**
     - **Both** — all selected wells, both merged and individual, will be plotted
     - **Merged Wells** — merges data from all selected wells into a single data point on the plot
     - **Individual Wells** — plots each well as a separate data point
   - **X Axis** — choose how the x axis labels are displayed and plotted, by sample name or by well number
   - **Error Model** — select Total Error Bars, Poisson Error Bars, or both
   - **Confidence Interval** — select either 95% or 68%; 95% is the default
2. **Options** – click the gear icon to bring up chart options menu.
   - **Scale** — choose auto or fixed
   - **Log** — change the axis to a logarithmic scale
   - **Range** — set minimum, maximum, and increment for y-axis and secondary y-axis (if chosen)
   - **Display** — toggle checkbox to choose whether to display Data Labels and Grid Lines
3. **Well selector** — select one or multiple wells to analyze in this well
4. **Data Table** — results for a selected well or group of wells, displayed by well number and sortable by any column by clicking on its header.


File
Plate Editor Tools
Analysis Tools
Plate Editor
Dashboard
Plate View
1D Amplitude
2D Amplitude
Concentration
Copy Number
Ratio
Droplets
Main Options
1
Show
● Both
● Merged Wells
○ Individual Wells
X Axis
☑ Well
☐ Sample
Error Model
☑ Total Error Bars
☑ Poisson Error Bars
Confidence Interval
○ 95%
● 68%
Ratio
1.04
B02
Options
2
Scale
○ Auto
● Fixed
Y Axis
☐ Log
Min
Max
Inc
☐ Fractional Abundance a/(a+b)
☐ Inverse
Display
☑ Data Labels
☑ Grid Lines
3
Well Data
4

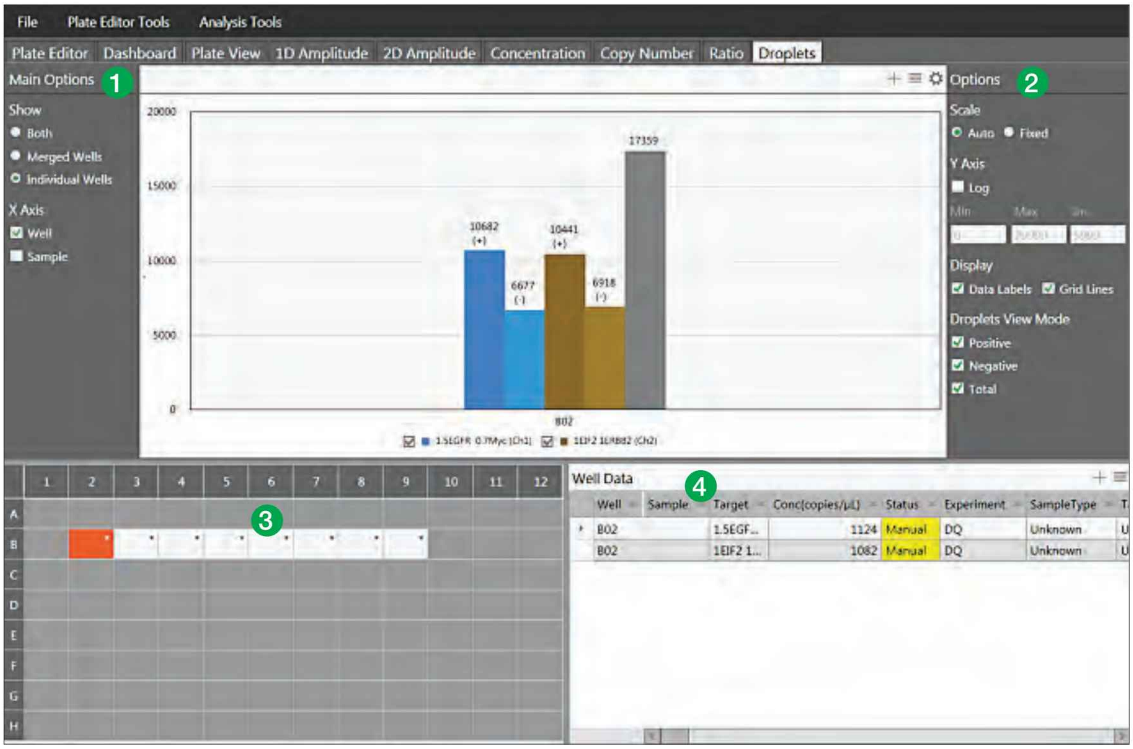
| Well | Sample | Target | Conc(copies/µL) | Status | Experiment | SampleType |
|---|---|---|---|---|---|---|
| B02 | | 1.5EGF... | 1124 | Manual | DQ | Unknown |
| B02 | | 1EIF2 1... | 1082 | Manual | DQ | Unknown |

Fig. 2.19. Ratio Tab.

## Legend Overview

This tab shows the ratio plot for individual or multiple wells and allows you to adjust plot parameters.

1. **Main Options**
   - **Show**
     - **Both** — all selected wells, both merged and individual, will be plotted
     - **Merged Wells** — merges data from all selected wells into a single data point on the plot
     - **Individual Wells** — plots each well as a separate data point
   - **X Axis** — choose how the x axis labels are displayed and plotted, by sample name or by well number
   - **Error Model** — select Total Error Bars, Poisson Error Bars, or both
   - **Confidence Interval** — select either 95% or 68%; 95% is the default
2. **Options** – click the gear icon to bring up chart options menu
   - **Scale** — choose auto or fixed
   - **Log** — change the axis to a logarithmic scale
   - **Range** — set minimum, maximum, and increment for y-axis
   - **Fractional Abundance a/(a + b)** — toggle checkbox to plot each unknown as a percent of the reference
   - **Inverse** — toggle checkbox to plot the reference as a percent of each unknown (the inverse fractional abundance)
   - **Display** — toggle checkbox to choose whether to display Data Labels and Grid Lines
3. **Well selector** — select one or multiple wells to analyze in this tab
4. **Data Table** — results for a selected well or group of wells, displayed by well number and sortable by any column by clicking on its header.


File
Plate Editor Tools
Analysis Tools
Plate Editor
Dashboard
Plate View
1D Amplitude
2D Amplitude
Concentration
Copy Number
Ratio
Droplets
Main Options
1
Show
● Both
● Merged Wells
○ Individual Wells
X Axis
☑ Well
☐ Sample
20000
15000
10000
5000
0
10682 (+)
6677 (-)
10441 (+)
6918 (-)
17359
B02
☑ 1.5EGFR 0.7Myc (Ch1)
☑ 1EIF2 1ERBB2 (Ch2)
Options
2
Scale
○ Auto
● Fixed
Y Axis
☐ Log
Min
Max
Inc
Display
☑ Data Labels
☑ Grid Lines
Droplets View Mode
☑ Positive
☑ Negative
☑ Total
1
2
3
4
5
6
7
8
9
10
11
12
A
B
C
D
E
F
G
H
3
Well Data
4

| Well | Sample | Target | Conc(copies/µL) | Status | Experiment | SampleType | T |
|---|---|---|---|---|---|---|---|
| B02 | | 1.5EGF... | 1124 | Manual | DQ | Unknown | U |
| B02 | | 1EIF2 1... | 1082 | Manual | DQ | Unknown | U |

Fig. 2.20. Droplets Tab.

## Legend Overview

This tab shows the droplets plot for one or more wells selected. Total droplets are shown by default for each well.

1. **Main Options**
   - **Show**
     - **Both** — all selected wells, both merged and individual, will be plotted
     - **Merged Wells** — merges data from all selected wells into a single data point on the plot
     - **Individual Wells** — plots each well as a separate data point
   - **X Axis** — select well name, sample name, or both
2. **Options** – click the gear icon to bring up chart options menu
   - **Scale** — choose auto or fixed
   - **Log** — change the axis to a logarithmic scale
   - **Range** — set minimum, maximum, and increment for y-axis
   - **Display** — toggle checkboxes to display Data Labels and Grid Lines
   - **Droplets View Mode** — toggle to choose any or all: Positive, Negative, Total
3. **Well selector** — select one or multiple wells to analyze in this tab
4. **Data Table** — results for a selected well or group of wells, displayed by well number and sortable by any column by clicking on its header.

## General Overview

### Keyboard Shortcuts

Keyboard shortcuts in QuantaSoft Analysis Pro are only usable in the Plate Editor Tab. After clicking on a tab: left and right arrows can be used to scroll through the tabs. In the Plate Editor Tab, after selecting a well: up, down, left, and right arrow keys can be used to scroll through the plate to select a well.

**Table 2. Keyboard shortcuts.**

| Keys | Function |
|---|---|
| Control + C | Copy contents of a well |
| Control + V | Paste copied well information |
| Control + Z | Undo last action |

### Rounding in QuantaSoft Analysis Pro

QuantaSoft Analysis Pro introduces a different set of rounding rules from the standard QuantaSoft software. Additional significant figures may be displayed in results.

- When the concentration call is >1,000, QuantaSoft displays 3 significant figures, while QuantaSoft AP rounds to the whole integer
- When the concentration call is <1, QuantaSoft displays two figures to the right of the decimal, while QuantaSoft AP displays three figures to the right of the decimal.

**Table 3. Examples of rounding in QuantaSoft as compared to QuantaSoft AP.**

| QuantaSoft | QuantaSoft AP |
|---|---|
| 3720.0 | 3723.0 |
| 4220.0 | 4218.0 |
| 0.67 | 0.675 |
| 1.12 | 1.12 |

## Experiment Types

QuantaSoft Analysis Pro has five different experiment analysis types, Direct Quantification, Copy Number Variation, Mutation Detection, Drop Off, and Gene Expression. Selecting an experiment type enables different assay options and analysis tools.

**Table X. Experiment types.**

| | |
|---|---|
| **Direct Quantification (DQ)** | Uses absolute quantification to determine the concentration (copies/µl) of target DNA copies in a sample and is the default experiment type.* |
| **Copy Number Variation (CNV)** | Determines concentration as with DQ and calculates the copy number of an unknown target relative to a known reference or references within the same well |
| **Mutation Detection (MUT)** | Determines concentration as with DQ and calculates the fractional abundance of an unknown mutant present at a low frequency in a wild-type background |
| **Drop Off (DOF)** | Determines absolute quantification of targets for assays designed to detect non-wild type sequences, such as indels and genome edits. The experiment type is designed to support an assay strategy where one probe counts all alleles and one “drop-off” probe sits on top of a predicted cut site. |
| **Gene Expression (GEX)** | Determines concentration as with DQ and calculates relative expression levels of an unknown target relative to a known reference or references within the same well. |

* QuantaSoft AP Software measures the number of positive and negative droplets for each target in a well. Droplets are assigned as positive or negative by thresholding based on their fluorescence amplitude. The software calculates the starting concentration of each target DNA molecule by modeling as a Poisson distribution; the formula used for Poisson modeling is: Copies per droplet = -ln(1-p) where p= fraction of positive droplets. Concentrations are provided in units of copies per microliter of input sample with 95% confidence intervals.

The experiment types created by QuantaSoft Standard Edition Software versions 1.4 or later will automatically be mapped (but can be changed) into QuantaSoft Analysis Pro as follows:

ABS **–>** DQ
RED **–>** DQ
CNV **–>** CNV
GEX **–>** GEX

## Assay Options

QuantaSoft Analysis Pro offers four assay configurations for varying levels and strategies of multiplexing targets within a well.

**Table X. Assay information.**

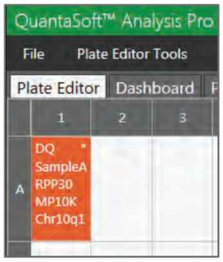
| | |
|---|---|
| **Simplex/duplex** | Experiment assuming one or two probe colors, FAM/EvaGreen and HEX/VIC and one or two targets per well, with a single target detected per channel. This is the default assay type and is available for DQ, CNV, MUT and GEX experiment types. |
| **Amplitude multiplex** | Method to increase multiplexing up to four targets per well, with one or two targets detected per channel. Available for DQ, CNV and GEX experiment types. |
| **Probe mix triplex** | Triplex mode that allows exactly three targets per well, with one target detected in each of two channels and a third target detected across both channels. Available for DQ, CNV and GEX experiment types. |
| **Advanced classification** | Method to increase multiplexing rate up to 20 targets per well in any combination of method targets detected per channel and requires manual classification of droplets. Available for DQ experiment type only |
| **Basic Drop-Off** | Exactly two targets per well, with the reference detected in both channels and the unknown detected in either one of the channels. Click on the Learn More link under Assay Details for setup help. Available for DOF experiment type only. |

## Experiment Examples

### Direct Quantification of Four (4) Targets in a Single Well by Amplitude Multiplexing

1. Open a file and go to the Plate Editor tab.
2. Select well(s) and set Experiment Type to Direct Quantification (DQ) at the top of the Edit Tools window to the right.

   **Note:** the default experiment type is DQ for .qlp files with ABS as the experiment configured in QuantaSoft 1.4 or later.
3. Click Apply to the right of the Experiment Type box in order to activate the experiment options in sections below.
4. Enter or modify sample name and type; the default sample type is Unknown. The supermix was assigned at the time of data collection and is not editable during data analysis. Click Apply to the right of the Sample Name.

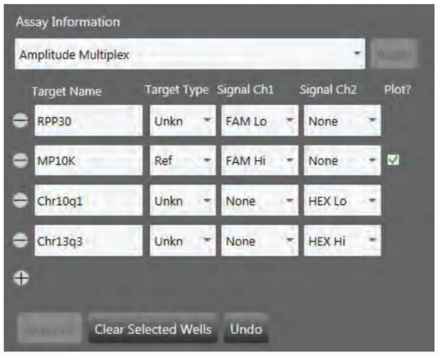


DQ *
SampleA
RPP30
MP10K
Chr10q1

**Fig. 2.21. Experiment type, sample name, and each target name are shown in the well once applied.** The * in the top right corner of the well indicates additional targets have been assigned to the well than what is visible on the screen. Viewall target information in the Edit Tools side panel for a selected well or wells.

5. Under Assay Information, select Amplitude Multiplex from the drop down menu and click Apply to the right.

   A maximum of 4 targets can be added by using the + icon at the bottom of the target list. Targets can be removed from the well by clicking the icons to the left of each target.
6. Enter target names and select the target type (unknown or reference) for each target in the well. Each target selected as a reference will enable ratio calculations of results. If no reference is selected, the target labeled with HEX/VIC channel 2 signal will be used as the default reference in calculating ratio values. If more than one reference is selected in the well, checkboxes appear to the right for using the reference in a geomean or single ratio calculation (Refer to second experiment example for using multiple references within a well).



Amplitude Multiplex

| Target Name | Target Type | Signal Ch1 | Signal Ch2 | Plot? |
|---|---|---|---|---|
| RPP30 | Unkn | FAM Lo | None | |
| MP10K | Ref | FAM Hi | None | ☑ |
| Chr10q1 | Unkn | None | HEX Lo | |
| Chr13q3 | Unkn | None | HEX Hi | |

**Fig. 2.22. Set Experiment Type to Direct Quantification (DQ) and enter target information.**

7. Assign each target an expected signal in either channel 1 (FAM/EvaGreen®) or channel 2 (HEX/VIC). Amplitude multiplex allows for up to two assays in each channel, indicated by Lo and Hi labels. In this multiplex mode, each target may have a signal for only one channel (for more on probe mixing, see Experiment Example number two).
8. Click Apply All at the bottom to confirm all well settings.

   **Note:** you can undo any well value that is applied by clicking the Undo button at the bottom, or by using Ctrl+Z on your keyboard. Wells can be copied and pasted onto other wells to expedite plate setup.
9. Once all wells are labeled, go to the 2D Amplitude tab to evaluate data and manually set thresholds. Automatic thresholding is not available for Amplitude Multiplexing experiments.

10. There are two options for setting the thresholds in the 2D plot. To use threshold lines, click the Threshold Line Mode button (pink crosshair) under Graph Tools to the left of the plot. Click anywhere on the plot to apply “best fit” threshold lines to the data. Adjust the threshold values by dragging the lines horizontally or vertically in the plot area, by entering a new threshold value in the boxes at the end of each threshold line, or by clicking the up and down arrows to incrementally adjust the numbers.

    If the data is not orthogonal and the threshold lines do not adequately divide the populations of droplets, use threshold clusters. Click any of the three Threshold Cluster Mode buttons (square, circle, or freehand). Draw the shape chosen around the cluster of droplets to be classified first. When you release the mouse button, a pop-up tool will appear to help you select the correct target combination. Target names are shown along the two axes based on the signal values assigned during plate setup. Click the colored box that best represents the location of the cluster of droplets selected to automatically classify the droplets as positive or negative for each given target.

11. The concentration results can be viewed in the data table below the plot area and in a concentration plot of the Concentration tab.

12. If one of your samples was identified as a Reference under Assay Information, the Ratio tab will provide a ratio of concentration for unknown versus the reference. For multiple references in a single well, see Experiment Example 2.

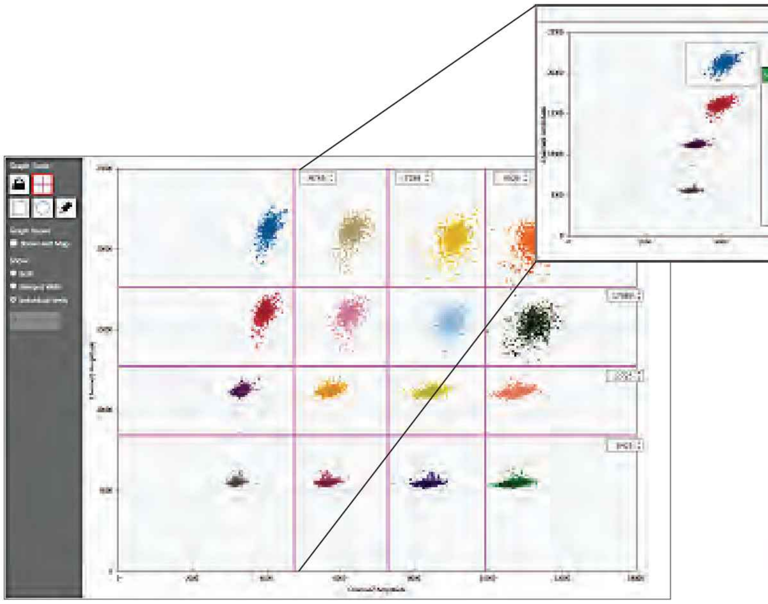
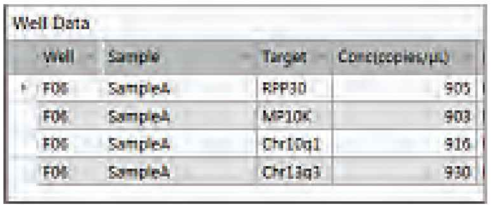


**Fig. 2.23. Threshold with lines.** Inset: Cluster with an automated pop-up tool to help you select the right target combination.



Well Data

| | Well | Sample | Target | Conc(copies/µL) |
|---|---|---|---|---|
| ▸ | F06 | SampleA | RPP30 | 905 |
| | F06 | SampleA | MP10K | 903 |
| | F06 | SampleA | Chr10q1 | 916 |
| | F06 | SampleA | Chr13q3 | 930 |

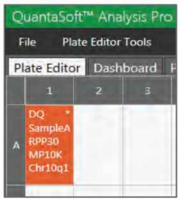
**Fig. 2.24. Concentrations displayed in data table.**

## Copy Number Variation with Probe Mix Triplex (Two Targets and a Reference)

1. Open a file and go to the Plate Editor tab.

2. Select well(s) and set Experiment Type to Copy Number Variation (CNV) at the top of the Edit Tools window to the right.

    **Note:** the default experiment type is CNV for .qlp files with CNV as the experiment configured in QuantaSoft 1.4 or later.



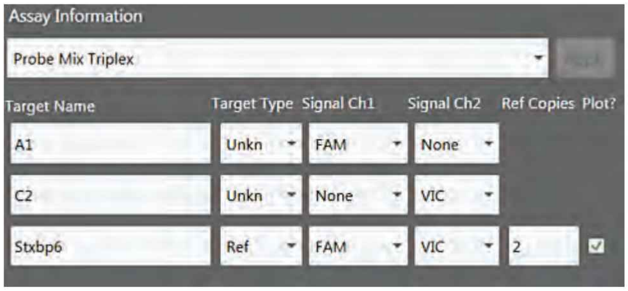
DQ
SampleA
RPP30
MP10K
Chr10q1

3. Click Apply to the right of the Experiment Type box in order to activate the experiment options in sections below.

4. Enter or modify sample name and type; the default sample type is Unknown. The supermix was assigned at the time of data collection and is not editable during data analysis. Click Apply to the right of the Sample Name.

5. Under Assay Information, select Probe Mix Triplex from the drop down menu and click Apply to the right. Only 3 targets will display and no additional targets can be added.

6. Enter target names and select the target type (unknown or reference) for each target in the well. Each target selected as a reference will enable ratio calculations of results. If no reference is selected, the target labeled with HEX/VIC channel 2 signal will be used as the default reference in calculating ratio values. If more than one reference is selected in the well, checkboxes appear to the right for using the reference in a geomean or single ratio calculation.



Assay Information

Probe Mix Triplex

| Target Name | Target Type | Signal Ch1 | Signal Ch2 | Ref Copies | Plot? |
|---|---|---|---|---|---|
| A1 | Unkn | FAM | None | | |
| C2 | Unkn | None | VIC | | |
| Stxbp6 | Ref | FAM | VIC | 2 | ☑ |

Fig. 2.25. Enter target information.

7. Assign the first and second target an expected signal in either channel 1 (FAM/EvaGreen®) or channel 2 (HEX/VIC), the third target should be assigned the signals from both channel 1 and channel 2. In this example the third target (channel 1 and channel 2 combined) is the reference. Assign the reference its correct Copy Number.

8. Click Apply All at the bottom to confirm all well settings.

   **Note:** you can undo any well value that is applied by clicking the Undo button at the bottom, or by using Ctrl+Z on your keyboard. Wells can be copied and pasted onto other wells to expedite plate setup.

9. Once all wells are labeled, go to the 2D Amplitude tab to evaluate data and manually set thresholds. Automatic thresholding is not available for Probe Mix Triplex experiments.

10. There is only one option for setting the thresholds for CNV experiments in the 2D plot. Click any of the three Threshold Cluster Mode buttons (square, circle, or freehand). Draw the shape chosen around the cluster of droplets to be classified first. When you release the mouse button, a pop-up tool will appear to help you select the correct target combination.Target names

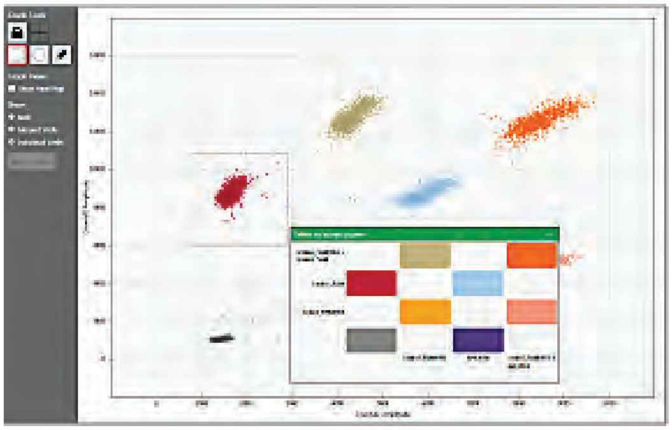
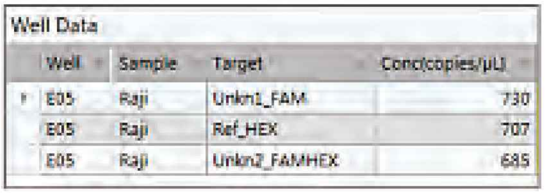


Fig. 2.26. Cluster with an automated pop-up tool to help you select the right target combination.

are shown along the two axes based on the signal values assigned during plate setup. Click the colored box that best represents the location of the cluster of droplets selected to automatically classify the droplets as positive or negative for each given target.



Well Data

| Well | Sample | Target | Conc(copies/µL) |
|---|---|---|---|
| E05 | Raji | Unkn1_FAM | 730 |
| E05 | Raji | Ref_HEX | 707 |
| E05 | Raji | Unkn2_FAMHEX | 685 |

Fig. 2.27. Concentrations displayed in data table.

11. The concentration results can be viewed in the data table below the plot area and in a concentration plot of the Concentration tab.

12. The copy number results can be viewed in the data table below the plot area and in a copy number plot of the Copy Number Tab.

## Genome Edit Detection using Drop-Off Assays

1. Open a file and go to the Plate Editor tab.

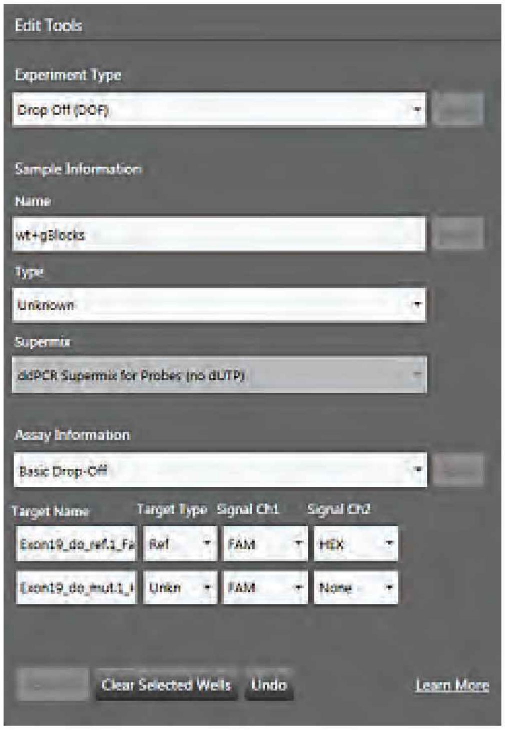
2. Select well(s) and set Experiment Type to Drop Off (DOF) at the top of the Edit Tools window to the right.

   **Note:** the default experiment type is DQ for .qlp files with ABS as the experiment configured in QuantaSoft 1.4 or later.

3. Click Apply to the right of the Experiment Type box in order to activate the experiment options in sections below.

4. Enter or modify sample name and type; the default sample type is Unknown. The supermix was assigned at the time of data collection and is not editable during data analysis. Click Apply to the right of the Sample Name.

5. Assay Information will automatically assign Basic Drop-Off. In this mode only two targets are available.



Edit Tools

Experiment Type: Drop Off (DOF)

Sample Information

Name: wt+gBlocks

Type: Unknown

Supermix: ddPCR Supermix for Probes (no dUTP)

Assay Information: Basic Drop-Off

| Target Name | Target Type | Signal Ch1 | Signal Ch2 |
|---|---|---|---|
| Exon19_do_ref.1_Fa | Ref | FAM | HEX |
| Exon19_do_mut.1_ | Unkn | FAM | None |

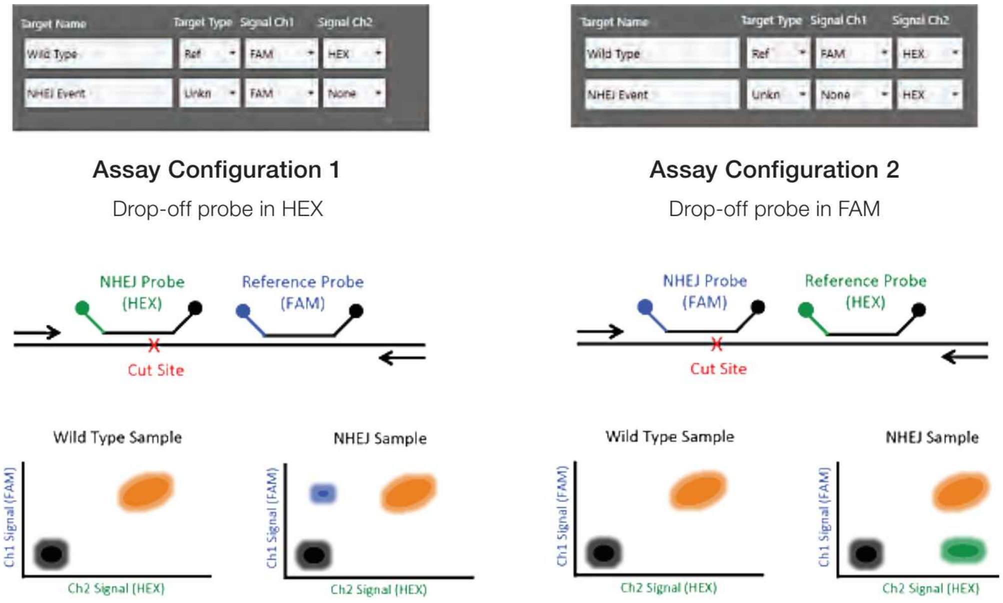
Fig. 2.28. Set Experiment Type to Drop Off (DOF) and enter target information. New users can click Learn More in the lower right-hand corner for contextual help.

6. Enter target names and select the target type (unknown or reference) for each target in the well. For this mode you should have one reference and one unknown. Each target selected as a reference will enable ratio calculations of results.

7. Assign the reference signals in both channel 1 (FAM/EvaGreen®) and channel 2 (HEX/VIC) and assign the unknown a signal in either channel 1 or channel 2, depending on the signal of the NHEJ probe.

8. Click Apply All at the bottom to confirm all well settings.

Two configurations of drop-off assays are possible and are shown below. Assign a signal (expected cluster position) to wild-type and NHEJ indel alleles. The signal produced by a wild-type allele will always be positive for both FAM and HEX, while the signal produced by an NHEJ indel allele depends on the assay configuration used (as shown below left and right).



| Target Name | Target Type | Signal Ch1 | Signal Ch2 |
|---|---|---|---|
| Wild Type | Ref | FAM | HEX |
| NHEJ Event | Unkn | FAM | None |

**Assay Configuration 1**

Drop-off probe in HEX

| Target Name | Target Type | Signal Ch1 | Signal Ch2 |
|---|---|---|---|
| Wild Type | Ref | FAM | HEX |
| NHEJ Event | Unkn | None | HEX |

**Assay Configuration 2**

Drop-off probe in FAM

**Fig. 2.29. Drop-Off Experiment setup for NHEJ quantification.** This information is available by clicking on the “learn more” link at the bottom right in the Edit Tools window.

**Note:** you can undo any well value that is applied by clicking the Undo button at the bottom, or by using Ctrl+Z on your keyboard. Wells can be copied and pasted onto other wells to expedite plate setup.

9. Once all wells are labeled, go to the 2D Amplitude tab to evaluate data and manually set thresholds. Automatic thresholding is not available for Drop Off experiments.

10. There is only one option for setting the thresholds for Drop Off experiments in the 2D plot. Click any of the three Threshold Cluster Mode buttons (square, circle, or freehand). Draw the shape chosen around the cluster of droplets to be classified first. When you release the mouse button, a pop-up tool will appear to help you select the correct target

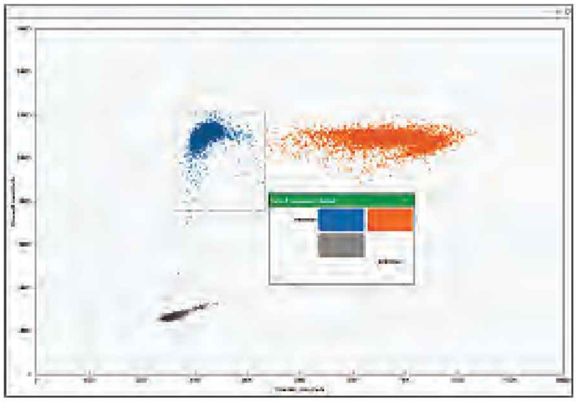
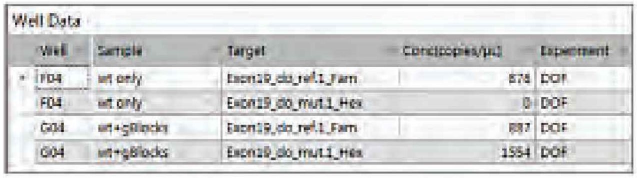


**Fig. 2.30. Cluster with an automated pop up tool to help you select the right target combination.**



Well Data

| Well | Sample | Target | Conc(copies/µL) | Experiment |
|---|---|---|---|---|
| F04 | wt only | Exon19_do_ref1_Fam | 878 | DOF |
| F04 | wt only | Exon19_do_mut1_Hex | 0 | DOF |
| G04 | wt+gBlocks | Exon19_do_ref1_Fam | 887 | DOF |
| G04 | wt+gBlocks | Exon19_do_mut1_Hex | 1554 | DOF |

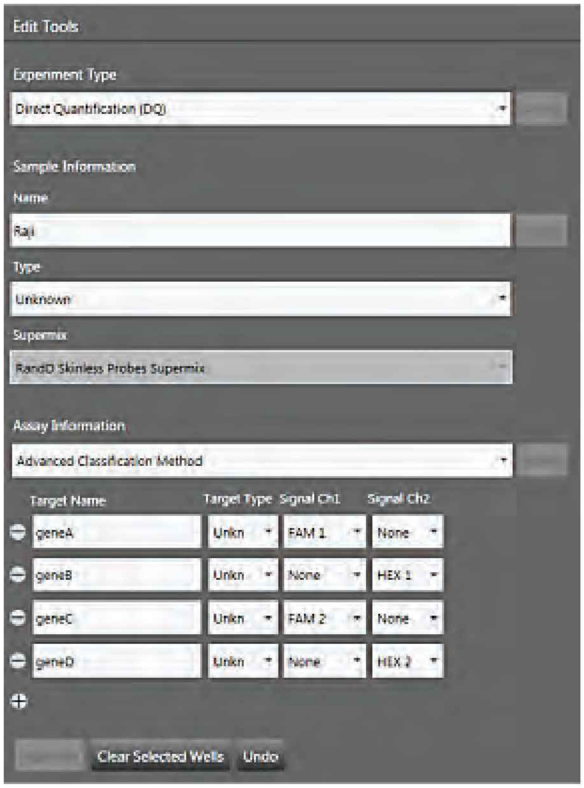
**Fig. 2.31. Concentrations displayed in data table.**

combination. Target names are shown along the two axes based on the signal values assigned during plate setup. Click the colored box that best represents the location of the cluster of droplets selected to automatically classify the droplets as positive or negative for each given target.

11. The concentration results can be viewed in the data table below the plot area and in a concentration plot of the Concentration tab.

## Advanced Classification Method

The Advanced Classification Method is intended for experienced users of Droplet Digital PCR. This analysis mode allows complete control of droplet identification and does not force or preassign any clusters. With that capability higher degrees of multiplexing can be accomplished through radial dye mixing. This capability comes with a draw back, multiple occupancy droplets become more difficult to identify so achieving a complete count of all targets might not be possible. The use of this mode for novice users is discouraged. This section will detail an example of utilizing this mode to accomplish multiplexing beyond what is offered through other assay modes.



Experiment Type: Direct Quantification (DQ)

Sample Information

Name: Raji

Type: Unknown

Supermix: RandD Skinless Probes Supermix

Assay Information: Advanced Classification Method

| Target Name | Target Type | Signal Ch1 | Signal Ch2 |
|---|---|---|---|
| geneA | Unkn | FAM 1 | None |
| geneB | Unkn | None | HEX 1 |
| geneC | Unkn | FAM 2 | None |
| geneD | Unkn | None | HEX 2 |

**Fig. 2.32. Set Experiment Type to Direct Quantification (DQ). Select Advanced Classification Method under Assay Information.**

1. Open a file and go to the Plate Editor tab.
2. Select well(s) and set Experiment Type to Direct Quantification (DQ) at the top of the Edit Tools window to the right.

   **Note:** the default experiment type is DQ for .qlp files with ABS as the experiment configured in QuantaSoft 1.4 or later.
3. Click Apply to the right of the Experiment Type box in order to activate the experiment options in sections below.
4. Enter or modify sample name and type; the default sample type is Unknown. The supermix was assigned at the time of data collection and is not editable during data analysis. Click Apply to the right of the Sample Name.
5. Under Assay Information, select Advanced Classification Method from the drop down menu and click Apply to the right. A maximum of 10 targets can be added by using the + icon at the bottom of the target list. Targets can be removed from the well by clicking the icons to the left of each target.
6. Enter target names and select the target type (unknown or reference) for each target in the well. Each target selected as a reference will enable ratio calculations of results. If no reference is selected, the target labeled with HEX/VIC channel 2 signal will be used as

the default reference in calculating ratio values. If more than one reference is selected in the well, checkboxes appear to the right for using the reference in a geomean or single ratio calculation

7. Assign the first target an expected signal in either channel 1 (FAM/EvaGreen®) or channel 2 (HEX/VIC). Assign your last target a signal in either channel 1 or channel 2, which was not selected for your first target. Each target in between will be assigned a signal in either channel 1 or channel 2 depending on mixture ratio of the target. Figure 2.32 shows examples of potential signal values

8. Click Apply All at the bottom to confirm all well settings.

   **Note:** you can undo any well value that is applied by clicking the Undo button at the bottom, or by using Ctrl+Z on your keyboard. Wells can be copied and pasted onto other wells to expedite plate setup.

9. Click the 2D Amplitude tab. All droplets are unclassified by default and will appear red. There is only one option for setting the thresholds for Advanced Classification experiments in the 2D plot. Use one of the Threshold Cluster Mode Tools (box, circle or freehand) to assign droplet clusters to Targets.

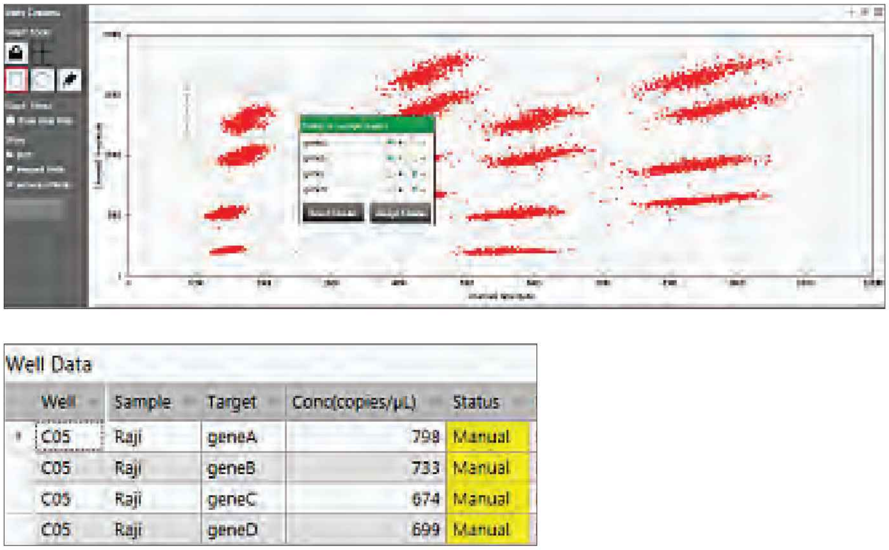
   Upon selection of droplet groups, a cluster assignment box will appear. Identify the composition of the cluster i.e. if the cluster is composed of positive droplets for Target-2, select the positive radial button for Target-2 and the negative radial buttons for the rest.

   The negative cluster should be described as negative for all the target genes and must be assigned to calculate concentrations.

   There must be at least one positive cluster for each target as well as the negative cluster assigned for calculations to take place. Unassigned droplets will be set aside for calculation purposes. If unsure of droplet/cluster composition, leave it unassigned (red).

10. The concentration results can be viewed in the data table below the plot area and in a concentration plot of the Concentration tab.

11. If one of your samples was identified as a Reference under Assay Information, the Ratio tab will provide a ratio of concentration for unknown versus the reference.



Well Data

| Well | Sample | Target | Conc(copies/µL) | Status |
|---|---|---|---|---|
| C05 | Raji | geneA | 798 | Manual |
| C05 | Raji | geneB | 733 | Manual |
| C05 | Raji | geneC | 674 | Manual |
| C05 | Raji | geneD | 699 | Manual |

**Fig. 2.33. Concentrations displayed in data table.**

# PCR Optimization Using Thermal Gradients

Optimizing the annealing temperature of your PCR assay is one of the most critical parameters for reaction specificity. Setting the annealing temperature too low may lead to amplification of nonspecific PCR products. On the other hand, setting the annealing temperature too high may reduce the yield of a desired PCR product. Even after calculating the $T_m$ of a primer, you may need to determine the optimal annealing temperature empirically because this temperature depends on buffer conditions as well as primer and probe concentrations. This involves repeating a reaction at many different temperatures. Similar time-consuming tests may also be required to optimize the sample denaturation temperature.

As with classical PCR reactions, the optimal annealing temperature for an assay can be easily determined using thermal cyclers that have a thermal gradient feature. The C1000 Touch™ Thermal Cycler offers a gradient feature. The gradient feature enables you to test a range of temperatures simultaneously, optimizing the annealing temperature in a single experiment.

To find the optimal annealing temperature for your reaction, test a range of temperatures above and below the calculated $T_m$ of the primers. The optimal annealing temperature is the one that results in the largest fluorescence amplitude difference between the positives and negatives and that avoids nonspecific amplification. It is also important to view the concentration values obtained across the thermal gradient simultaneously when considering the fluorescence amplitude separation of positives and negatives. A sample annealing temperature optimization experiment is shown in Figure 2.34 demonstrating separation of positives and negatives of replicate samples across the thermal gradient.

For two-color assays, a thermal gradient can be used to identify a temperature where both assays perform well.

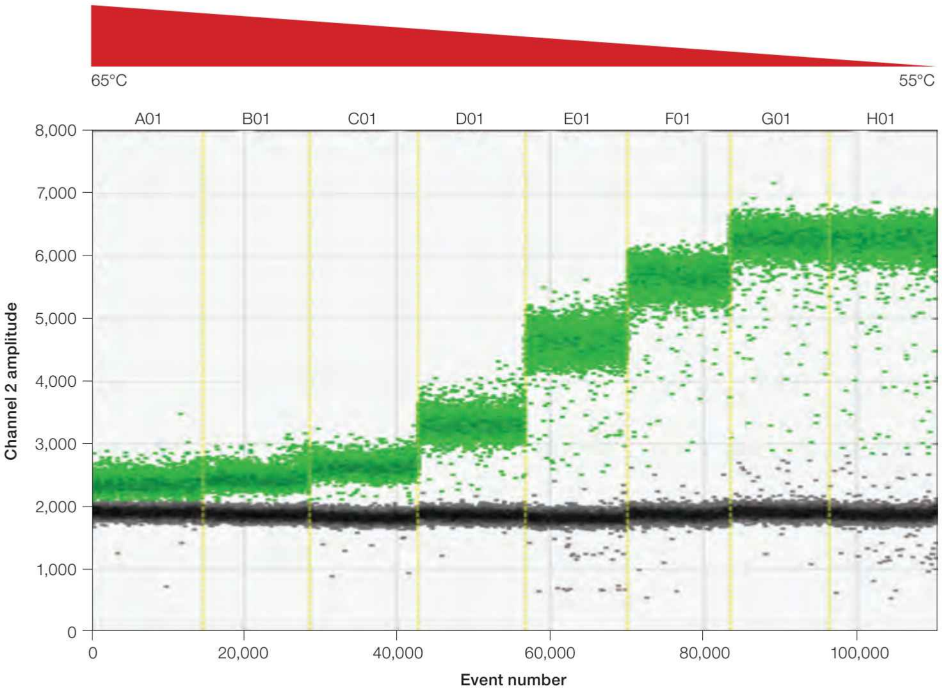


Fig. 2.34. Thermal gradient optimization.

# ddPCR Using the QX200 System and EvaGreen® dsDNA Dye

The QX200 System can measure amplified DNA using Bio-Rad's QX200™ ddPCR™ EvaGreen® Supermix, template, and a pair of primers. EvaGreen® dsDNA binding dye is similar to SYBR® Green in that it fluoresces upon binding double-stranded DNA (Figure 2.35).

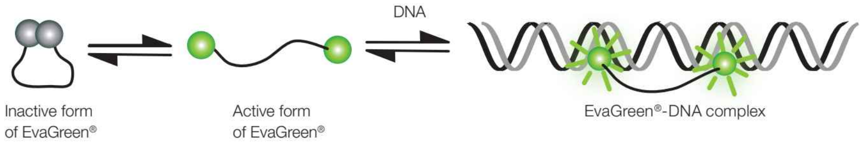
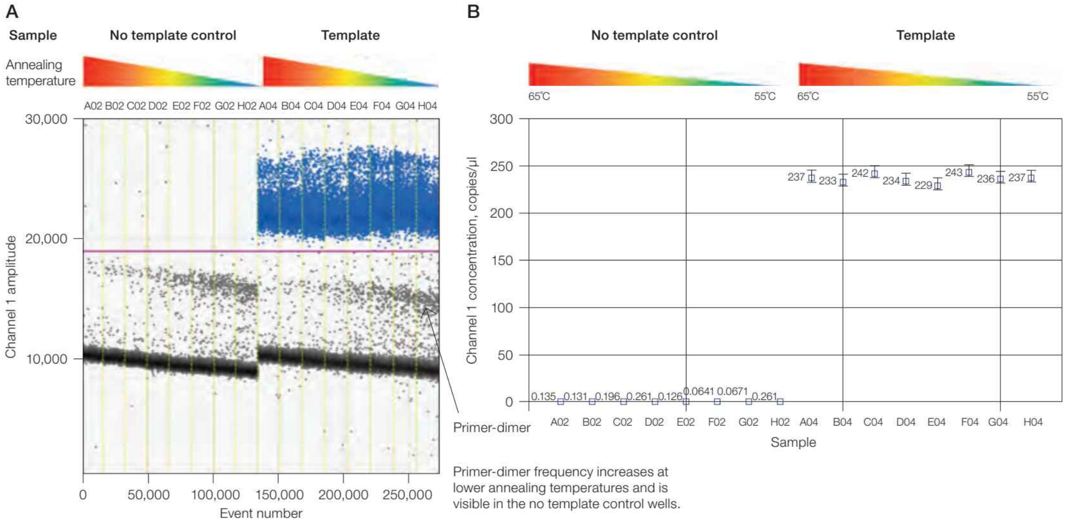


**Fig. 2.35. EvaGreen® dye binds to dsDNA via a "release-on-demand" mechanism.**

EvaGreen® dsDNA binding dye enables double-stranded DNA detection with the convenience and savings of only needing primers to amplify and detect product with the added high-resolution features of ddPCR. ddPCR with EvaGreen® can be used with applications such as gene expression, copy number variation, DNA rearrangement detection, micro RNA detection, and multiplexing.

The fluorescence amplitude of each droplet with EvaGreen® varies with amplicon length and with PCR efficiency. Longer amplicons will bind to more EvaGreen® dye molecules and therefore be brighter. PCR reactions that are not at their most efficient annealing/extension temperature will result in fewer amplicons in each positive droplet at end point and therefore lower fluorescence amplitude. Despite the range in positive fluorescence amplitude, accurate quantification is still routinely achieved. These features provide the user the ability to measure length or PCR efficiency as well as the ability to measure multiple targets in the same well while only using primers. Additionally, primer-dimers and off-target amplicons can be detected as low fluorescence amplitude droplets. With the EvaGreen® supermix reactions it is possible to simply set the threshold above the primer-dimer in order to obtain an accurate quantification of the target without having to redesign the primers (Figure 2.36).



Primer-dimer frequency increases at lower annealing temperatures and is visible in the no template control wells.

**Fig. 2.36. Accurate quantification of target is obtained using Bio-Rad's QX200 System with the QX200™ ddPCR™ EvaGreen® Supermix. A**, threshold set above primer-dimer; **B**, accurate quantification of the target is obtained without redesigning primers.

In Figure 2.37, a gradient of annealing/extension temperatures is shown for three amplicons with different lengths: A, B, and C correlate to 200, 99, and 62 bp amplicons, respectively. The experiment demonstrates the effect amplicon length has on fluorescence amplitude as well as how annealing/extension temperature affects amplitude and the ability to resolve products with different lengths based on amplicon size. Red arrows show increasing separation between 200 and 99 bp amplicons as the annealing/extension temperature is lowered from 65 to 55˚C.

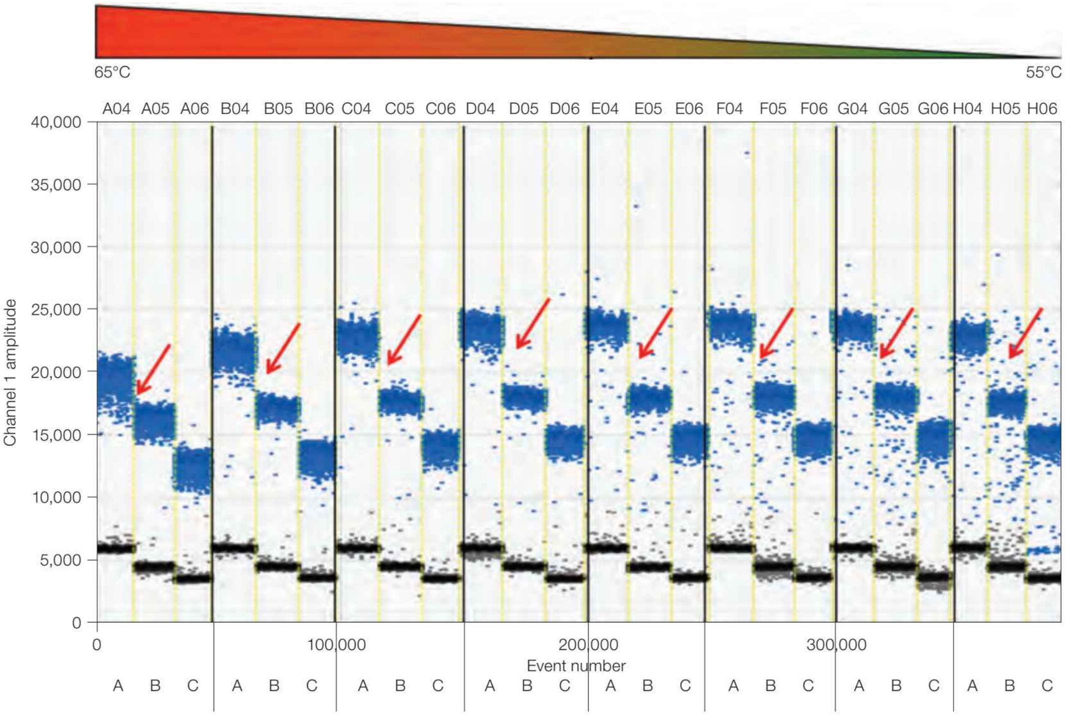


**Fig. 2.37. Gradient of annealing/extension temperatures for three amplicons with different lengths: A (200 bp), B (99 bp), and C (62 bp).**

## EvaGreen® and Gene Expression

Measuring gene expression using ddPCR and EvaGreen® allows you to detect and quantify splice variants as well as contaminating genomic DNA. Figure 2.38 shows an experiment demonstrating that splice variants can be seen as a cluster of droplets with higher fluorescence amplitude, referred to as a super cluster. Control samples with no reverse transcriptase added were run to verify that the super cluster was not due to residual genomic DNA.

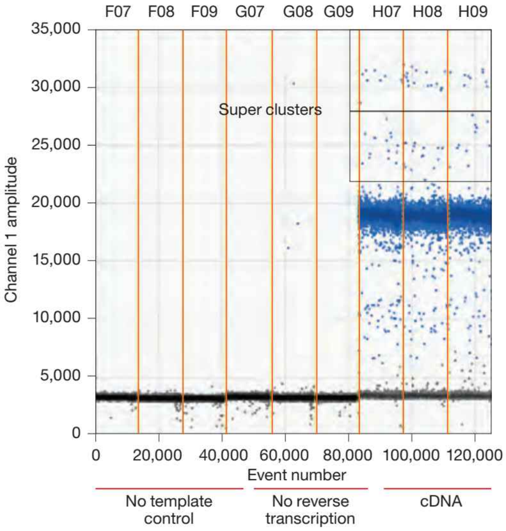


**Fig. 2.38. Splice variant discrimination using the QX200™ ddPCR™ EvaGreen® Supermix with a single set of primer pairs.**

## Multiplexing with EvaGreen®

Differences in droplet amplitude due to differences in amplicon length or optimal annealing temperature allow for multiplexing in a single well. Figure 2.39 shows a 2-D fluorescence plot containing two amplicons, where differences in amplitude are observed due to differences in optimal annealing temperature. Primer set 1 has an optimal $T_m$ of 63°C, therefore droplets having this target have relatively high fluorescence amplitudes at 63°C. Primer set 2 has an optimal annealing/extension temperature of 59°C and therefore has a much lower fluorescence amplitude at 63°C. Distinct separation of these clusters allows a copy number to be calculated with high precision (Figure 2.39). Similarly, varying amplicon length instead of PCR efficiency can be used to measure multiple targets in the same well. However, when using amplicon length to multiplex with EvaGreen® it is important to use an optimal annealing/extension temperature for thermal cycling (Figure 2.37).

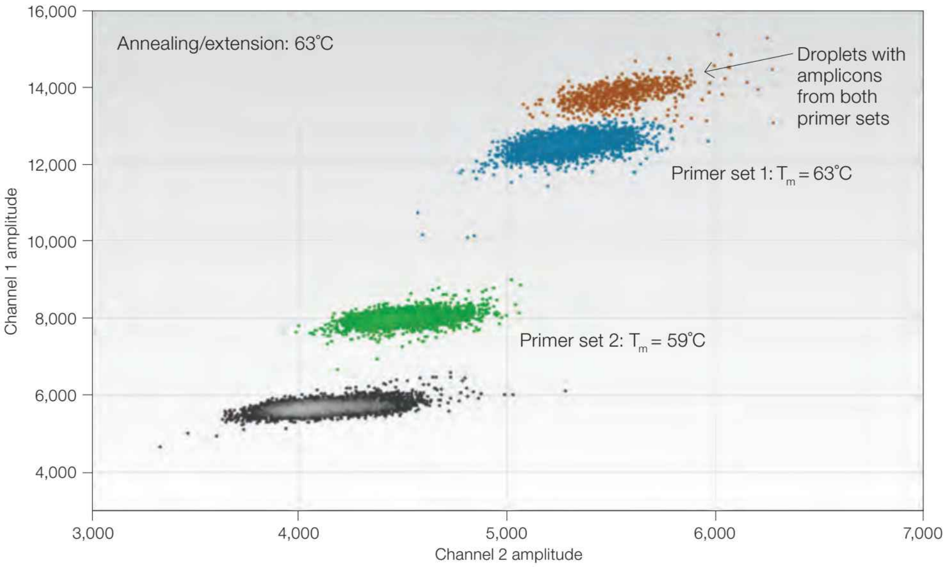


**Fig. 2.39. Multiplex assays performed using ddPCR with EvaGreen®.** Differences in amplitude are due to differences in optimal annealing temperature. $T_m$, melting temperature.

# Reference

Untergasser et al. (2007). Primer3Plus, an enhanced web interface to Primer3. Nucleic Acids Res 35 (web server issue), W71–W74.

# 3 Absolute Quantification and the Statistics of Droplet Digital™ PCR

## Running Absolute Quantification Experiments

Absolute quantification (ABS) is fundamental to all Droplet Digital PCR (ddPCR™) applications. Partitioning template DNA into uniform droplets enables highly quantified measurements of target DNA using the QX200™ or QX100™ Droplet Digital PCR System and the appropriate automatic statistical analysis in QuantaSoft™ Software. ddPCR does not require a standard curve, and this simplifies the experimental planning and procedures, and reduces result variability and cost.

Important ABS experiment considerations are as follows:

- Digest the template DNA to fragment the genome. Select restriction enzymes that do not cut either the target or reference amplicons
- The restriction-digested DNA should be at a higher concentration in order to have a lower concentration in the master mix

**Note:** Account for the concentration of the template in any restriction digest and the amount of template in the final 1x ddPCR supermix to backcalculate the original concentration of the stock template.

**Note:** Do not exceed 5,000 copies of target/µl of the final ddPCR reaction mix.

- Use a thermal gradient to optimize ddPCR results
- In the Well Editor of QuantaSoft Software, designate ABS for the experiment type
- In the Well Editor, indicate which assay is the target and which assay is the reference when performing multiplex reactions

## Absolute Quantification Data Analysis

The ABS experiment is designed to quantify the concentration of the target and give a result in copies/µl of the final 1x ddPCR reaction.

If the plate was set up for ABS analysis, automatic thresholding determines concentrations and populates the data tables in the analysis mode of the software. The threshold may be manually adjusted. Note specifically any wells that are flagged as No Call in the status column of the data tables.

QuantaSoft Software will return a No Call for wells with too many positive droplets (not enough empty droplets to apply Poisson statistics), wells with Quality Scores below 0.85, and wells with fewer than 10,000 droplets. After visually inspecting the data, you may set a threshold manually and QuantaSoft Software will complete the calculations.

Figure 3.1 shows excellent separation between positive droplets (green) and negative droplets (black) in the chart on the left, showing droplets (event number) vs. fluorescence amplitude. The histogram on the right plots amplitude vs. the frequency of the populations of droplets and can assist in setting the threshold.

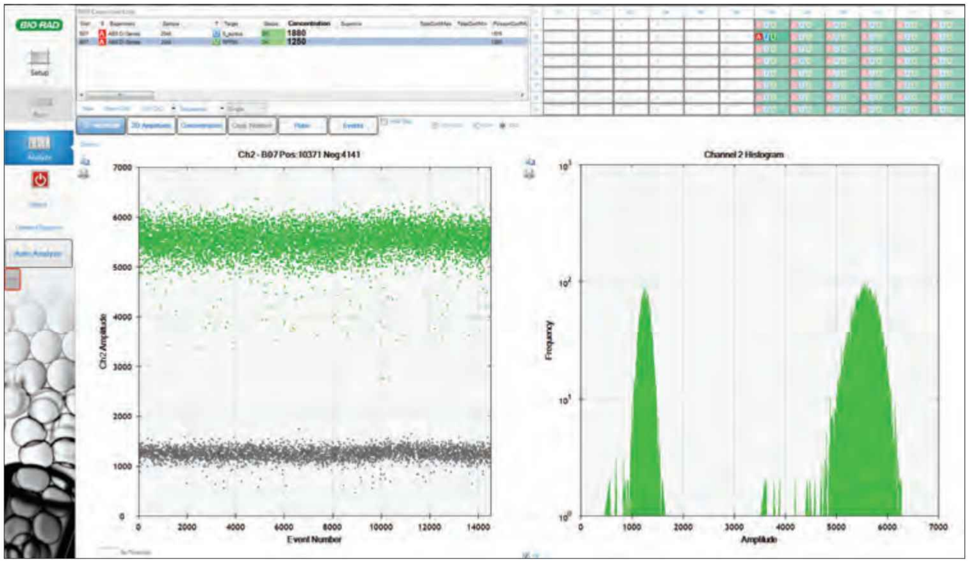

BIO-RAD
Setup
Analyze
Auto Analyze
Concentration
1880
1250
2D Amplitude
Concentration
Plate
Events
Ch2 - B07 Pos:10371 Neg:4141
Ch2 Amplitude
7000
6000
5000
4000
3000
2000
1000
0
0
2000
4000
6000
8000
10000
12000
14000
Event Number
Channel 2 Histogram
Frequency
$10^3$
$10^2$
$10^1$
$10^0$
0
1000
2000
3000
4000
5000
6000
7000
Amplitude

Fig. 3.1. Example of a well-performing assay for absolute quantification.

QuantaSoft Software measures the number of positive and negative droplets for each fluorophore in each sample. It then fits the fraction of positive droplets to a Poisson algorithm to determine the starting concentration of the target DNA molecule in units of copies/µl input from the sample (Figure 3.2).

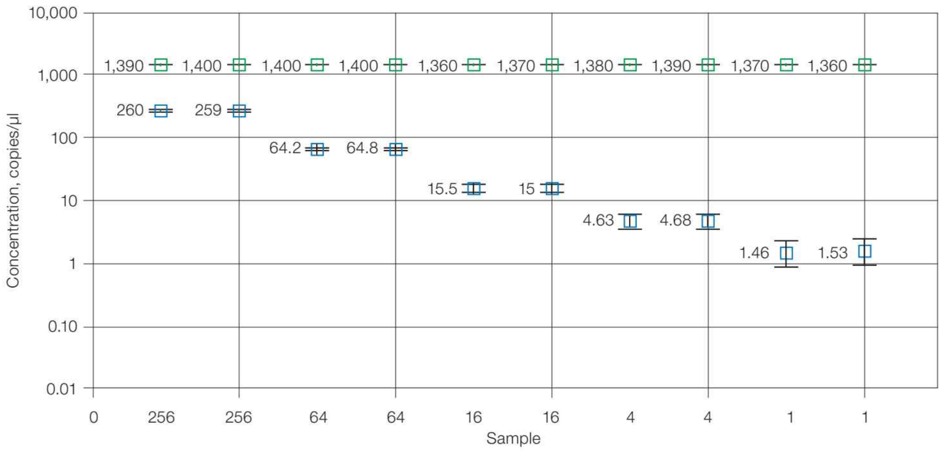


**Fig. 3.2. Sample concentrations are plotted as copies/µl from the sample.**

# Statistics of ddPCR

In ddPCR, DNA molecules and PCR reagents are partitioned into droplets. Some droplets will contain one or more copies of the target DNA molecule (DNA fragment that contains primer and probe binding sites for a given assay) and some will not contain any copies of the target. Droplets are subjected to PCR amplification and the targeted PCR product, with an associated fluorescence signal, is generated in droplets that initially contained the target DNA. The concentration of the target species in the original sample is determined from the fraction of droplets that have a fluorescence signal.

In ddPCR, target molecules are distributed randomly into droplets. This assumption is at the heart of all digital PCR approaches. Random partitioning means a given target molecule is equally likely to end up in any of the 20,000 droplets. Random partitioning also means target molecules move independently of each other and do not interact with each other.

The droplet generator partitions samples into approximately 20,000 droplets of identical volume (about 1 nl). Some droplets are lost in transfer steps and others are eliminated by the stringent metrics applied by QuantaSoft Software as the droplets pass through the Droplet Reader, resulting ultimately in data from 12,000–16,000 droplets being used in subsequent concentration calculations. Reading only a subset of the total droplets has no impact on the concentration measurement, because the concentration is calculated based on the fraction of droplets that is empty (that is, the fraction that does not contain any target DNA).

As an example, suppose a sample contains 1,000 copies/µl of a target molecule, and the sample is loaded into two ddPCR wells. In one well, only 10,000 droplets are read by the software, and in the other, 15,000 droplets are read. In both cases, the fraction of empty droplets will be the same, and thus the concentration in copies/µl will be the same in both wells (within the limits of the instrument measurement error).

At the extreme ends of the concentration range (for example, fewer than ten copies of target in a well or more than 120,000 copies of target in a well), fewer droplets in a well will lead to slightly larger error bars.

**Note:** For simplicity, we will use 20,000 partitions in the examples in this chapter.

## Copies per Microliter

QuantaSoft Software provides concentration results in copies of target per microliter (copies/µl). The dynamic range of ddPCR extends from fewer than 0.25 copies/µl to more than 5,000 copies/µl. In many cases, the fundamental quantity of interest to a user is the number of copies of target in the starting sample.

The following example shows how copies/µl is converted to copies in the starting material.

Mix 10 µl of sample with 12.5 µl of ddPCR Supermix for Probes and 2.5 µl of assay (primer and probe mix), for a total volume of 25 µl. Load 20 µl of this mix into a DG8™ Droplet Generator Cartridge and run ddPCR. The software reports that the concentration is 8 copies/µl. Two equivalent methods illustrate how many total copies and how many copies/µl of the target DNA were present in the original 10 µl sample.

- Method #1: The ratio of sample to total volume is 10/25 = 2/5. Since there were 8 copies/µl in the final PCR mix, there were 8 x (5/2) = 20 copies/µl in the original sample. In the full 10 µl of the original sample, there were 10 x 20 = 200 copies of the target DNA
- Method #2: Since there were 8 copies/µl in the PCR mix and a total of 25 µl of the PCR mix was made, there were 8 x 25 = 200 copies of the target DNA in the PCR mix. This mix contained 10 µl of the original sample, so there were 200 copies of target DNA in the full 10 µl of starting sample, and 200/10 = 20 copies/µl of target in the starting sample

## Copies per Droplet

A useful unit for discussing the calculations underlying ddPCR is copies of target per droplet (CPD). For illustration purposes in this chapter we will use 1 nl volume.

CPD represents a number per unit volume, and not a mass per unit volume. CPD is the average number of target copies/droplet. For a CPD of 2, for example, some droplets will have 0 copies, some will have 1, 2, 3, and so on.

There are multiple ways to calculate CPD:

- Example 1:

CPD = total number of molecules/total number of droplets

If you have 100,000 molecules total and 20,000 droplets, then

CPD = 100,000 molecules/20,000 droplets

CPD = 5 molecules/droplet

- Example 2:

CPD = (molecules/µl) x droplet volume (µl)

If you have 20 molecules in a 20 µl sample with 1 nl droplet volume, then

20 molecules/20 µl = 1.0 molecule/µl

CPD = (1.0 molecule/µl) (0.001 µl/droplet)

CPD = 0.001

## Low Concentration Example

When there are far fewer molecules than partitions (for example, 500 molecules or less in 20,000 partitions), it is relatively easy to see how the ddPCR approach enables accurate quantification. Figure 3.3 shows a sample that contains six target DNA molecules. If the entire sample is converted into droplets, we will expect to find exactly six droplets that contain template, since it is statistically highly unlikely that a single droplet will start with more than one target molecule.

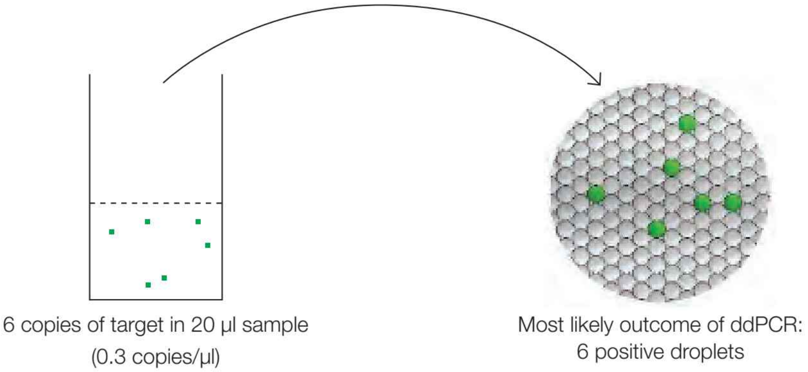

6 copies of target in 20 µl sample
(0.3 copies/µl)
Most likely outcome of ddPCR:
6 positive droplets

**Fig. 3.3. Outcome of ddPCR in two modes of analysis.**

For 20,000 droplets, the following concentration calculation is used (assumes an approximate droplet volume of 1 nl or $10^{-3}$ µl).

$$\text{Volume analyzed} = 20{,}000 \times 10^{-3}\ \mu\text{l/droplet} = 20\ \mu\text{l}$$

$$\text{Concentration} = \frac{\text{Copies of target}}{\text{Volume analyzed}} = \frac{6\ \text{copies}}{20\ \mu\text{l}} = 0.3\ \text{copies}/\mu\text{l}$$

**Note:** The final concentration is the same if we analyze 10,000 droplets and observe three copies of target:

$$\text{Volume analyzed} = 10{,}000 \times 10^{-3}\ \mu\text{l/droplet} = 10\ \mu\text{l}$$

$$\text{Concentration} = \frac{\text{Copies of target}}{\text{Volume analyzed}} = \qquad = 0.3\ \text{copies}/\mu\text{l}$$

## Intermediate Concentration Example

Consider the case in which there are 5,000 target molecules in 20,000 droplets (5,000 targets in 20 µl = 250 copies/µl = 0.25 CPD). Random partitioning of target molecules into droplets will lead to some droplets with 2, 3, or even 4 copies, and correspondingly more than 75% of the droplets will have zero copies. Poisson statistics tells us exactly how many droplets to expect in each category. Table 3.1 shows the expected percentage of droplets in each category. Note that in ddPCR, each droplet is distinguished as either empty/negative (zero targets) or occupied/positive (one or more targets). For the 0.25 CPD case, 78% (not 75%) of the droplets will be negative and 22% (not 25%) will be positive on average. Since Poisson statistics arise in multiple different contexts in ddPCR, we describe this phenomenon as “partitioning statistics.”

**Table 3.1. Expected percentage of droplets containing target DNA copies.**

| Targets | Droplets, % |
|---|---|
| 0 | 78 |
| 1 | 19.5 |
| 2 | 2.4 |
| 3 | 0.2 |
| 4 | 0.01 |

## High Concentration Example

Consider the case in which 50,000 target molecules are present in 20 µl (50,000 targets in 20 µl = 2,500 targets/µl = 2.5 CPD). With an average of 2.5 copies of target/droplet, it’s not immediately obvious that there will be any empty droplets. But Poisson statistics predicts that there will be some empty droplets (1,642 empty droplets) and gives a precise relationship between the average number of copies/droplet and the expected fraction of empty droplets. Table 3.2 shows how many droplets we expect to see with 0, 1, 2, 3, 4, or more copies/droplet when there are on average 2.5 copies of target/droplet, or 50,000 copies in a 20 µl reaction volume.

**Table 3.2. Expected percentage of droplets containing the target at high concentration.**

| Number of Target Molecules | Count of Droplets | Total Droplets, % |
|---|---|---|
| 0 | 1,642 | 8.21 |
| 1 | 4,101 | 20.5 |
| 2 | 5,130 | 25.7 |
| 3 | 4,275 | 21.4 |
| 4 | 2,672 | 13.4 |
| 5 | 1,336 | 6.68 |
| 6 | 557 | 2.78 |
| 7 | 199 | 0.99 |
| 8 | 62 | 0.31 |
| 9 | 17 | 0.086 |
| 10 | 4 | 0.02 |
| 11 | 1 | 0.0049 |

Even at an average of 5 copies/droplet (the upper end of the recommended loading range), we expect to see about 134 empty droplets in a total of 20,000 droplets (data not shown).

## Looking across the Whole Concentration Range

Figure 3.4 shows the number of droplets with 0, 1, 2, 3 (and so on) copies of the target DNA at different DNA concentrations. There is a simple mathematical relationship between the fraction of droplets that are unoccupied (black bar) and the concentration of target molecules. As the concentration of input target DNA increases, the expected number of unoccupied droplets decreases.

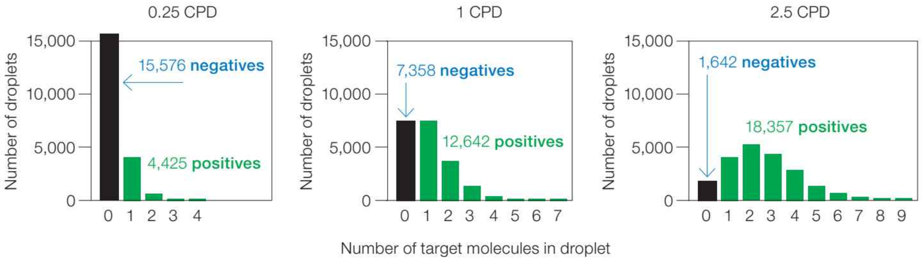


**Fig. 3.4. Number of droplets with copies of target DNA at different DNA concentrations.** CPD, copies per droplet.

**Note:** Quantification is impossible when there are no unoccupied droplets, which is approached at 8 CPD.

## Concentration Calculation

### Definitions

$C$ = copies per droplet (CPD)

$E$ = observed fraction of empty droplets

$V_{droplet}$ = volume of droplet

$N_{neg}$ = number of negative droplets

$N$ = total number of droplets

## Formula for Calculating Concentration

The formula used by QuantaSoft Software to calculate concentration is:

$$\text{Concentration} = -\ln\left(\frac{N_{neg}}{N}\right)/V_{droplet}$$

## Derivation of Concentration Formula

The Poisson distribution gives probability Pr(*n*) that a droplet will contain *n* copies of target if the mean number of target copies per droplet is *C*:

$$\Pr(n) = \frac{C^{n}e^{-c}}{n!}$$

Inputting $n = 0$ gives the probability that a droplet will be empty for a given value of *C*:

$$\Pr(0) = e^{-c}$$

For a large number of droplets, the observed fraction of empty droplets (*E*) gives a very good estimate of Pr(0), so:

$$E = e^{-c}$$

Solving for *C* gives:

$$C = -\ln(E)$$

Recall that *C* is copies per droplet. To convert to copies per µl, divide by the droplet volume:

$$\text{Concentration} = \frac{C}{V_{droplet}}$$

Combining the two previous equations gives:

$$\text{Concentration} = \frac{-\ln(E)}{V_{droplet}}$$

Note that by definition:

$$E = \frac{N_{neg}}{N}$$

Combining the two equations above gives:

$$\text{Concentration} = -\ln\left(\frac{N_{neg}}{N}\right)/V_{droplet}$$

$$C = -\ln\left(\frac{N_{neg}}{N}\right)$$

or

$$C = \ln(N) - \ln(N_{neg})$$

## Errors in ddPCR

Two types of errors are reported by QuantaSoft Software: technical errors (Poisson errors) and total errors.

Technical errors (Poisson errors): a measurement error based on known properties of the system that can be calculated based on a single well or by pooling all the droplets from multiple wells. One of the assumptions in this error calculation is that the sample in a ddPCR well is a subsample from a larger whole. Poisson errors are an excellent estimate of technical replicate measurement errors. A technical replicate in this context is when aliquots of the same sample are loaded into multiple ddPCR wells. An interesting and useful feature of ddPCR is that it is possible to estimate the technical replicate error from a single concentration measurement.

Total errors: the recommended error to use in most applications in biology. It is the greater of the technical error and the standard error of the mean. This method is the preferred one because it prevents underestimation of the error. We can say with certainty that if you observe a standard error of the mean that is less than the theoretical technical error, you were just “lucky” and the true error is in reality at least as big as the technical error.

For experiments with replicates, both an empirical error measurement (the total error) and a theoretical technical replicate error (the Poisson error or technical error) are calculated. In most cases, it is more appropriate to report the total error measurement. If the wells are true technical replicates as defined above, the total error and the Poisson error will be nearly identical for good assays.

**Note:** Error bars are shown in QuantaSoft Software as 95% confidence intervals. The closest parallel in quantitative PCR (qPCR) is the mean ± 2 times the standard error of the mean. Some qPCR Systems by default show 68% confidence intervals, or the mean ± 1 times the standard error of the mean.

**Note:** The theory behind technical errors is explained in Appendix B.

# 4 Copy Number Variation Analysis

## Overview

Analysis of copy number (CN) involves determining the number of copies of a given target locus with respect to an invariant reference locus. An alteration in copy number state with respect to the reference locus is copy number variation (CNV). CNV could be a deletion or duplication of a locus with respect to the number of copies of the reference locus (and hence genomes) present in the cell.

CNV is a prominent source of interindividual variability in the human genome, and CNV has been associated with cancers, neurological and autoimmune diseases, and adverse drug response. The major technical challenge in CN assessment is the ability to discriminate, with statistical confidence, between consecutive CN states (Figure 4.1). Fundamentally, as CN state increases, the percentage difference in target genomic material between states decreases. For example, for a given target locus, a CN of 3 is 50% more abundant in concentration per genome than a CN of 2, while a CN of 5 is only 25% more abundant per genome than a CN of 4, and so on. Current methods to analyze CNV, including single nucleotide polymorphism (SNP)–based microarrays, comparative genomic hybridization, and quantitative PCR (qPCR), lack the sensitivity and resolution needed for this fine degree of quantitative discrimination in CNV analysis.

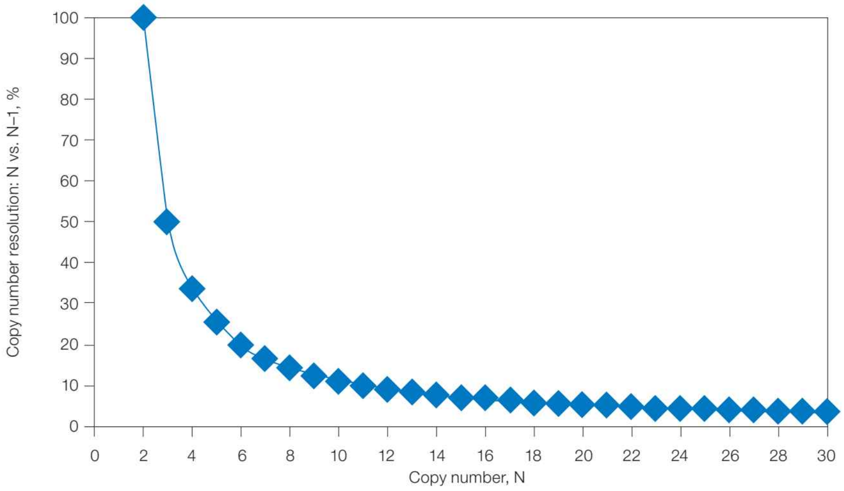


**Fig. 4.1. Discrimination between consecutive CN states is more difficult at higher order copy numbers.**

The massive partitioning of a CNV Droplet Digital™ PCR (ddPCR™) reaction across up to 20,000 droplets enables the fine quantitative discrimination required to resolve consecutive copy number states beyond CN 3. This is due to the high precision of the ddPCR concentration measurement and the absolute nature of the measurement. For example, a 20% difference in target concentration between a CN 5 vs. CN 6 sample can be reproducibly resolved using ddPCR (Figure 4.2). This discrimination is both a function of concentration differences between consecutive CN states, as well as the amount of DNA assayed per well.

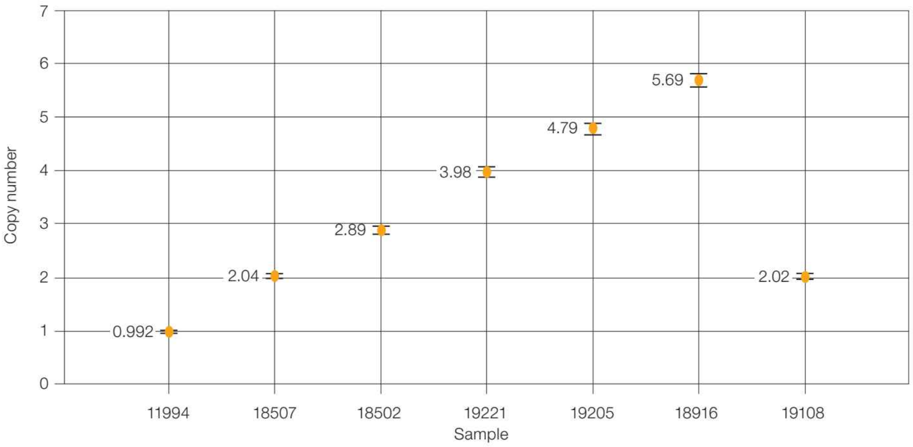


**Fig. 4.2. ddPCR measurements of *MRGPRX1* copy numbers across seven Coriell DNA samples.**

# CNV Calculations

CNV analysis by ddPCR involves quantification of target and reference loci through the use of duplex target and reference assays. In QuantaSoft™ Software, copy number is determined by calculating the ratio of the target molecule concentration to the reference molecule concentration, times the number of copies of reference species in the genome (usually 2).

$$CN = \frac{A}{B} N_B$$

$A$ = concentration of target species

$B$ = concentration of reference species

$N_B$ = number of copies of reference loci in the genome (usually 2)

The error bars on a CN estimate in QuantaSoft Software are the 95% confidence interval of this measurement.

# CNV Analysis in Homogeneous Samples

Analysis of CNV in homogeneous samples involves evaluation of CN state from a sample source where every cell is presumed to be identical in CN state. Examples include *CYP2D6* genotyping in patient samples, trisomy 21 detection in a clonally derived Down syndrome cell line, and counting transgene copies in a genetically modified organism (GMO) plant stock. The challenge in homogeneous samples is largely a function of the level of discrimination required at a high CN state to differentiate between adjacent CN states.

For some applications, for example, binning cancer patient samples by oncogene amplification status, discrimination between high CN states (for instance, 50 vs. 51 copies) may not be as critical as discriminating between CN states of 2 vs. 10 vs. 50.

# CNV Analysis in Heterogeneous Samples

Some sample types are heterogeneous for CN state — meaning that for a given sample of cells, some have altered CN status and some do not. Examples include a breast cancer biopsy, where only a fraction of the cells are amplified for *HER2*, detection of fetal trisomy 21 from a maternal blood sample, or somatic mosaicism in normal tissue. In heterogeneous samples, the ability to accurately quantify CN alterations is a function of both the percentage of assayed cells with a CN alteration and the increase in CN state of the target gene in those cells. It is more challenging to detect CNV for a target locus in a sample where 10% of the cells have CN 3 instead of 2 than in a sample where 10% of the cells have a CN of 10 instead of 2. The problem becomes more difficult as the percentage of cells with the CN alteration decreases. Detection of CN variant cells in a heterogeneous sample is a function of both the magnitude of the CN alteration and the rarity of the altered cell (Table 4.1).

**Table 4.1. Detection of CN variant cells in a heterogeneous sample.**

| Sensitivity Required for CN Determination in a Heterogeneous Sample | | | |
|---|---|---|---|
| | Diploid Copy Number | | |
| Cells with CN Alteration, % | Wild Type | Amplified | Discrimination Needed, % |
| 10 | 2 | 3 | 5 |
| | 2 | 10 | 40 |
| | 2 | 50 | 240 |
| 1 | 2 | 3 | 0.5 |
| | 2 | 10 | 4 |
| | 2 | 50 | 24 |
| 0.1 | 2 | 3 | 0.05 |
| | 2 | 10 | 0.4 |
| | 2 | 50 | 2 |
| 0.01 | 2 | 3 | 0.005 |
| | 2 | 10 | 0.040 |
| | 2 | 50 | 0.240 |

# Planning CNV Experiments

## Assay Design

For CNV ddPCR, target and reference assays are designed with different probe fluorophores, generally FAM-target and HEX-reference (or VIC-reference).

When possible, target and reference assays should have amplicons of similar length. This is particularly important for fragmented samples, like material extracted from formalin-fixed, paraffin-embedded (FFPE) tissue. Because CNV calculates the ratio of target concentration to reference concentration, if either target or reference amplicon site in the genome is under-represented due to fragmentation, the ratio call will be off-integer.

Check for secondary (nonspecific) products using the UCSC In-Silico PCR site (**http://genome.ucsc.edu/cgi-bin/hgPcr?command=start**). This is particularly important for CN-variable targets or members of gene families because of the presence of highly similar pseudogenes or homologs.

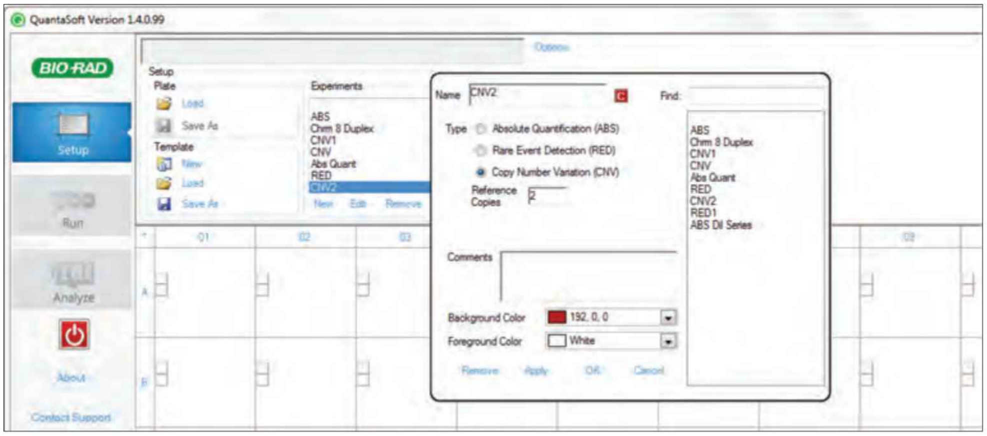
An ideal reference assay yields expected concentrations reproducibly and robustly across standard reaction parameters, duplex partners, and sample sources. A reference assay should be CN invariant. A good reference assay performs well across a wide range of annealing/extension temperatures.

Examples of human genome reference loci:

- *RPP30* (NCBI gene 10556)
- Ultraconserved sequences (PMID: 15131266)

## Running a CNV Assay

Select **Copy Number Variation (CNV)** as the experiment type in QuantaSoft Software when loading wells. Double click on the experiment name in the main software window to set the ploidy for the reference (Figure 4.3).


QuantaSoft Version 1.4.0.99
BIO-RAD
Setup
Run
Analyze
About
Contact Support
Options
Setup
Plate
Load
Save As
Template
New
Load
Save As
Experiments
ABS
Chrm 8 Duplex
CNV1
CNV
Abs Quant
RED
CNV2
New
Edit
Remove
01
02
03
02
A
B
Find:
ABS
Chrm 8 Duplex
CNV1
CNV
Abs Quant
RED
CNV2
RED1
ABS Dil Series
Remove
Apply
OK
Cancel

Name: CNV2

Type:
○ Absolute Quantification (ABS)
○ Rare Event Detection (RED)
● Copy Number Variation (CNV)

Reference Copies: 2

Comments:

Background Color: 192, 0, 0

Foreground Color: White

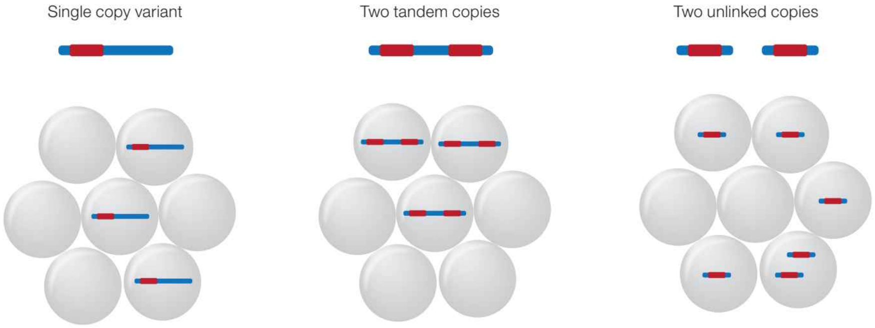
Fig. 4.3. Setting the ploidy for the CNV reference.

**Note:** R2 means 2 copies/genome (diploid) or 1 copy/haploid genome. If CNV experimental type is not selected for your wells, the CNV tab will not be available. This affects only the CNV charting calculations and can be applied during setup or any time after data collection.

For the reference assay, select **Reference** as type. Put this assay's concentration as the denominator, for example, "b" for the ratio chart and for the fractional abundance chart, and assign the CN selected to this assay. If both Ch1 and Ch2 are selected as "unknown," Ch1 is automatically "a" and Ch2 is automatically both "b" and the CNV reference.

## Restriction Digestion

To achieve optimal accuracy in CN measurements, restriction digestion of genomic DNA is required. Restriction digestion separates tandem gene copies, ensuring proper random partitioning into droplets (Figure 4.4). Restriction digestion can also reduce sample viscosity and improve assay performance by improving template accessibility.


Single copy variant
Two tandem copies
Two unlinked copies

3 positive droplets

3 positive droplets: inaccurate CNV estimate

5 positive droplets: accurate CNV estimate

Fig. 4.4. Restriction digestion separates tandem gene copies.

Considerations in planning a restriction digestion.

- Do not cut the target or reference amplicon
- Choose a methylation-insensitive enzyme
- Read the chosen restriction enzyme FAQs on the manufacturer's website. They often describe known issues such as star activity
- For most assays, a fragment size of 5 kb or less works fine. This can typically be achieved with a 4-cutter or 6-cutter enzyme. For some assays, smaller template fragment lengths (<500 bp) are required. This may be due to nearby inhibitory secondary DNA structures/elements that get cut away with a smaller fragment size
- RestrictionMapper (**www.restrictionmapper.org**) is a website that can help you determine template fragment length and whether your designed amplicon is cleaved by a given restriction enzyme
- For human genomic DNA, use 10 U restriction enzyme/µg DNA
    - Up to 20 U enzyme/µg DNA is acceptable. Higher concentrations of enzyme could be required to resolve higher CN targets
- Some ddPCR reactions are inhibited when high volumes of digestion buffer are included because of high salt concentrations
    - If possible, avoid use of NEBuffer 3 and 3.1, which have the highest salt concentration
    - Digested templates should be diluted a minimum of 10-fold in the final ddPCR reaction setup to reduce the final salt concentration in ddPCR. For example, no more than 2 µl of a 1x digest reaction should be loaded into a 20 µl ddPCR reaction if possible
    - Many assays will perform well regardless of which NEBuffer is used or the amount of 1x digest reaction loaded into ddPCR
- Incubate the reaction for 1 hr at the recommended temperature
- Heat inactivation is not required, but can be considered if long-term storage of digested template is required. **Do not heat inactivate at greater than 65°C**
- DNA purification is not necessary after restriction digestion
- Store digested DNA at –20°C

Lists of recommended restriction enzymes for CNV ddPCR are provided in Tables 4.2 and 4.3. Conditions for a typical restriction enzyme digestion are in Table 4.4. For more information, visit the New England Biolabs, Inc. website (**www.neb.com**).

**Table 4.2. Recommended restriction enzymes for CNV ddPCR (most-preferred 4-cutters).**

| Restriction Enzyme | Sequence | Digestion Buffer (old) | Digestion Buffer (new) | Incubation Temperature, °C |
|---|---|---|---|---|
| CviQI | G/TAC | NEBuffer 2, 3, BSA | 3.1 | 25 |
| MseI | T/TAA | NEBuffer 2, 4, BSA | CutSmart | 37 |
| AluI | AG/CT | NEBuffer 1, 2, 4 | CutSmart | 37 |
| HaeIII | GG/CC | NEBuffer 2, 4 | CutSmart | 37 |
| BsmI | GAATGC(1/–1) | NEBuffer 4 | CutSmart | 65 |
| BstYI | R/GATCY | NEBuffer 2,4 | 2.1 | 60 |

BSA, bovine serum albumin.

**Table 4.3. Recommended restriction enzymes for CNV ddPCR (most-preferred 6-cutters).**

| Restriction Enzyme | Sequence | Methylation Sensitivity | Digestion (old) | Digestion Buffer (new) | Incubation Temperature, °C |
|---|---|---|---|---|---|
| HindIII | A/AGCTT | None | NEBuffer 2 | NEBuffer 2.1 | 37 |
| EcoRI | G/AATTC | CpG | NEBuffer 1, 2, 3, 4 | EcoRI buffer | 37 |

NEB, nuclear extraction buffer.

**Table 4.4. Conditions for a typical restriction enzyme digestion.**

| Reaction Component | Final Concentration | Volume (50 µl/reaction), µl |
|---|---|---|
| 10x digestion buffer | 1x | 5 |
| gDNA | 100 ng/µl | Variable |
| Restriction endonuclease | 10 U/µg DNA | Variable |
| 100x BSA (as needed) | 1x | 0.5 |
| Water | — | Variable |

BSA, bovine serum albumin; gDNA, genomic DNA.

Newly designed assays should be run across a thermal gradient (for example, 65–55°C) to identify an annealing/extension temperature that optimizes separation between positive and negative droplets while minimizing rain (droplets that fall between the major positive and negative populations). If possible, select an annealing/extension temperature that optimizes performance of both target and reference assays.

## DNA Loading for Lower-Order CN Analysis (diploid CN <10)

For most routine CNV ddPCR applications, where a diploid target CN is expected to be 10 or less, approximately 0.2–1.0 reference gene copies per droplet (CPD) of DNA sample should be loaded per well. This corresponds to 10–66 ng of human genomic DNA per well.

## DNA Loading for Higher-Order CN Analysis (diploid CN >10)

When target CN is expected to exceed 10 copies/diploid genome, <15 ng of human genomic DNA should be loaded per well. This ensures both target and reference copies fall within the dynamic range of the instrument (Table 4.5). For example, if a reference locus is loaded at 1 CPD (1,000 copies/µl), a target present 20 times more frequently per genome is effectively loaded at 20 CPD (20,000 copies/µl), which is beyond the range of accurate quantification of the instrument. If a reference locus is loaded at 0.2 CPD (200 copies/µl), a target present 20 times more frequently is loaded at 4 CPD (4,000 copies/µl), which is within the dynamic range of the instrument. For diploid CN >50, strategies where multiple wells are used to estimate target and reference concentrations can be used.

**Table 4.5. Sample loading considerations for higher-order CN targets (10–50 copies/diploid genome).**

| | | Target Loading for CN ≥10 | | |
|---|---|---|---|---|
| **Reference Loading for CN 2, CPD** | **Reference, copies/µl** | **Target, copies/µl** | | |
| | CN 2 | CN 10 | CN 20 | CN 40 |
| 1 | 1,000 | 5,000 | 10,000 | 20,000 |
| 0.2 | 200 | 1,000 | 2,000 | 4,000 |

CPD, copies per droplet; CN, copy number.

# 5 Rare Mutation and Sequence Detection

## Overview

Droplet Digital™ PCR (ddPCR™) enables detection and analysis of nucleic acids at a level of sensitivity and precision beyond the capabilities of previous methods. Applications that focus on the lower limits of nucleic acid detection can be separated into two classes:

- Rare mutation detection
- Rare sequence detection

Rare mutation detection (RMD) and rare sequence detection (RSD) can be classified according to their assay components. An RMD assay is made up of a single primer set plus two competitive probes (Figure 5.1). RSD uses either a single assay to detect a rare sequence per unit volume (Case 1) or two independent, noncompetitive assays to detect a rare sequence as a proportion of a more common sequence (Case 2). Both RMD and RSD take advantage of the high sensitivity of ddPCR.

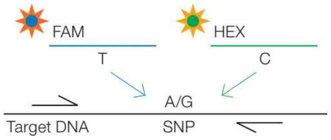
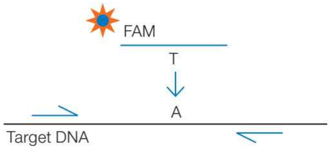
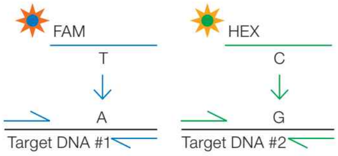
| | Measurement Units | Examples | Assay Components | Assay Schematic |
|---|---|---|---|---|
| **Rare Mutation Detection** | | | | |
| | Ratio (% or a/[a+b]) | ■ Tumor biopsy: 0.01% mutant detected, or 1 in 10,000<br>■ Single nucleotide polymorphisms (SNPs)<br>■ Small indels | 1 primer pair, 2 competitive probes |  |
| **Rare Sequence Detection** | | | | |
| Case 1 | Copies/volume | ■ Invasive species monitoring (copies/ml lake water)<br>■ Human immuno-deficiency virus (HIV) detection (copies/ml plasma) | 1 assay (1 primer/probe set) |  |
| Case 2 | Ratio (% or a/[a+b]) or copies/unit reference | ■ Gene expression of rare transcripts<br>■ Viral staging<br>■ Indels | 2 independent assays (2 primer/probe sets) |  FAM<br>HEX<br>T<br>C<br>A<br>G<br>Target DNA #1<br>Target DNA #2 |

**Fig. 5.1. Rare mutation detection and rare sequence detection assays.**

For any rare detection assay, standard design rules should be used in the design of TaqMan probes and primer sets (see Chapter 2). In general, primers should be designed to have melting temperatures ($T_m$) of ~60°C (1 M NaCl, 1 µM concentration) and should be within 2°C of each other. Furthermore, the probe must have a melting temperature 3–10°C higher than the primer $T_m$. A temperature binding enhancer, such as a locked nucleic acid (LNA) can be used to shorten the number of nucleotides in the probe while maintaining a higher $T_m$. Finally, the mutant site should be positioned near the middle of the probe sequence.

Rare detection assay designs should be validated with a temperature gradient to ensure the highest specificity between the mutant and wild-type clusters. The optimal annealing temperature is defined by the condition in which the mutant probe exhibits no false positives in the wild-type–only sample and the relative distance between the FAM-only (mutant) and HEX (or VIC)-only (wild-type) clusters is maximal.

To enable the ultrasensitive detection of mutant targets, very high loads of DNA are required. However, for ddPCR, amounts of intact human DNA exceeding 66 ng (20,000 genome equivalents)/20 µl reaction negatively affect the accuracy of DNA quantification. To mitigate this effect, gDNA must be fragmented by restriction digestion using an enzyme that cuts around the amplicon(s) of interest. Once fragmented, the human gDNA concentration can exceed 1 µg/20 µl reaction without affecting DNA quantification.

# Rare Mutation Detection

Rare mutation detection occurs when a biomarker exists within a background of a highly abundant counterpart that differs by only a single nucleotide. Many methods for mutation analysis have poor selectivity and fail to detect mutant sequences with abundances of less than one in 100 wild-type sequences (Scott 2011, Benlloch et al. 2006, Whitehall et al. 2009). If enough DNA sample is available for testing, a limit of detection of 1 mutant in 100,000 wild type can be detected using a well-designed assay and proper experimental setup.

## ddPCR for Rare Allele Detection and Experimental Considerations

The partitioning effect, which is a hallmark of ddPCR technology, has an important impact on the sensitivity and specificity of a PCR reaction. For applications reliant on measuring a low-abundance rare mutant allele in a large excess of wild-type DNA, partitioning the sample into droplets increases the sensitivity by orders of magnitude by effectively diluting away the background. This means that the mutant target is present in droplets at a much greater relative abundance than it would be in bulk solution. Considering a rare allele detection scenario where the desired mutant has an abundance of 0.1% relative to wild-type DNA, Figure 5.2 shows that randomly partitioning the sample into 20,000 1-nl droplets increases the relative abundance by 330-fold, resulting in drastic improvement of the achievable sensitivity compared to real-time PCR.

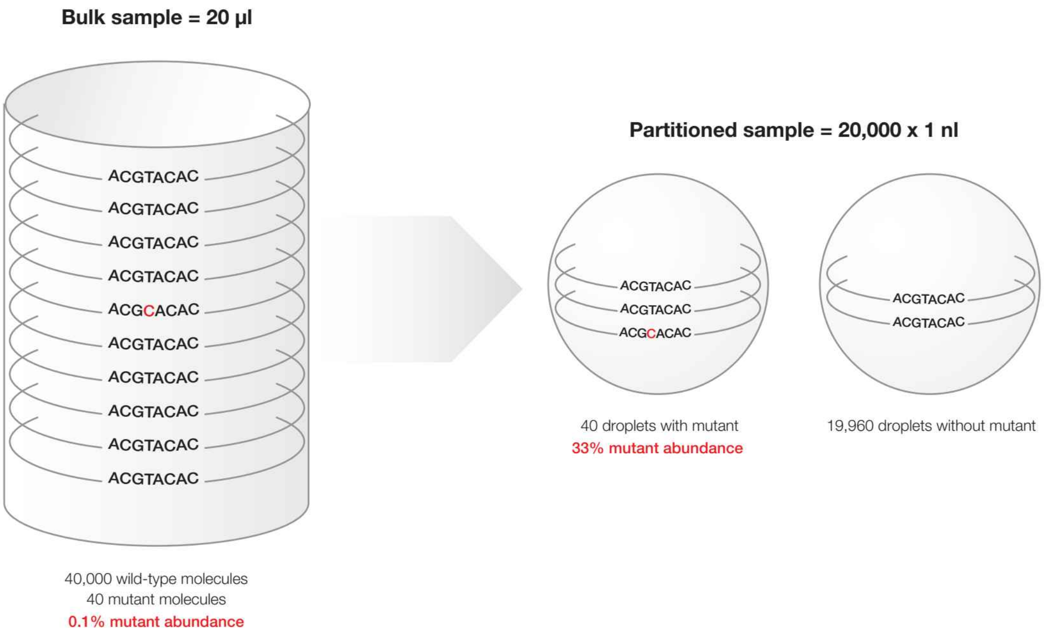

Bulk sample = 20 µl
ACGTACAC
ACGTACAC
ACGTACAC
ACGTACAC
ACGCACAC
ACGTACAC
ACGTACAC
ACGTACAC
ACGTACAC
ACGTACAC
40,000 wild-type molecules
40 mutant molecules
0.1% mutant abundance
Partitioned sample = 20,000 x 1 nl
ACGTACAC
ACGTACAC
ACGCACAC
40 droplets with mutant
33% mutant abundance
ACGTACAC
ACGTACAC
19,960 droplets without mutant

**Fig. 5.2. Effect of partitioning on the relative abundance of a mutant target in an excess of wild-type DNA.**

Figure 5.2 depicts a bulk 20 µl solution containing 40 mutants and 40,000 wild-type DNA molecules with a mutant abundance of 0.1%, which is beyond the limit of quantification by real-time PCR. After random partitioning into 20,000 1-nl droplets, there are 40 droplets containing target molecules at a relative mutant abundance of 33%, which are easily distinguishable from the 19,960 droplets containing wild-type molecules only.

## RMD Experiment Considerations

The first consideration for low-level detection is the amount of DNA available. If 1 mutant in 100,000 wild-type sequences, or 0.001%, is to be detected, then statistically at least 300,000 haploid genomes must be screened. For human DNA this is 1 µg of DNA.

The challenge for RMD assay development is that it must discriminate between two highly similar sequences, one of which is significantly more abundant than the other. An example of an RMD assay is detection of a single nucleotide polymorphism (SNP) in a cancer biopsy. An RMD assay comprises a single set of primers plus two competitive probes (each probe with a different fluorophore), one detecting the wild-type allele, and one detecting the variant allele.

## Testing an RMD Assay

RMD assays should be tested for specificity by first running a temperature gradient at a relatively high wild-type DNA concentration and then by running a concentration gradient of wild-type DNA with a spike-in of mutant DNA to assess specificity.

First, run a temperature gradient using both a row/column of restriction digested, 100% wild-type DNA at a concentration between 2,000–5,000 copies/µl previously restriction digested and a row/column of 2,000–5,000 copies/µl wild-type DNA including 50–200 copies/µl mutant DNA. Using the 1-D amplitude plot, select the $T_m$ with no false positives, good separation between positive and negative populations, and minimal probe cross-reactivity (Figure 5.3). Also, be sure to simultaneously check the concentration plots to make sure the concentration is the expected result at the temperature selected.

In Figure 5.3, wells A–H are an annealing/extension gradient from 65–55°C, respectively. Panel A is a 1-D amplitude plot showing the FAM-labeled mutant assay results from a temperature gradient on the SNP *PI3Kca_E545K*. Wells A03–H03 contain 100% wild-type DNA, and A04–H04 contain 50% wild-type/mutant DNA. Well D was selected at an annealing/extension temperature of 61.4°C. At lower annealing/extension temperatures, false positives appear. Panel B is a 1-D amplitude plot of FAM-labeled mutant assay results of *PI3Kca_H1047R*. Wells A06–H06 contain 100% wild-type DNA, and A07–H07 contain 50% wild-type/mutant DNA. Well B was selected at an annealing/extension temperature of 64.5°C. At lower annealing/extension temperatures, the positive clusters for the VIC channel have increasing probe cross-reactivity with the nonspecific target, causing the wild-type droplets to have some level of FAM fluorescence in the FAM channel.

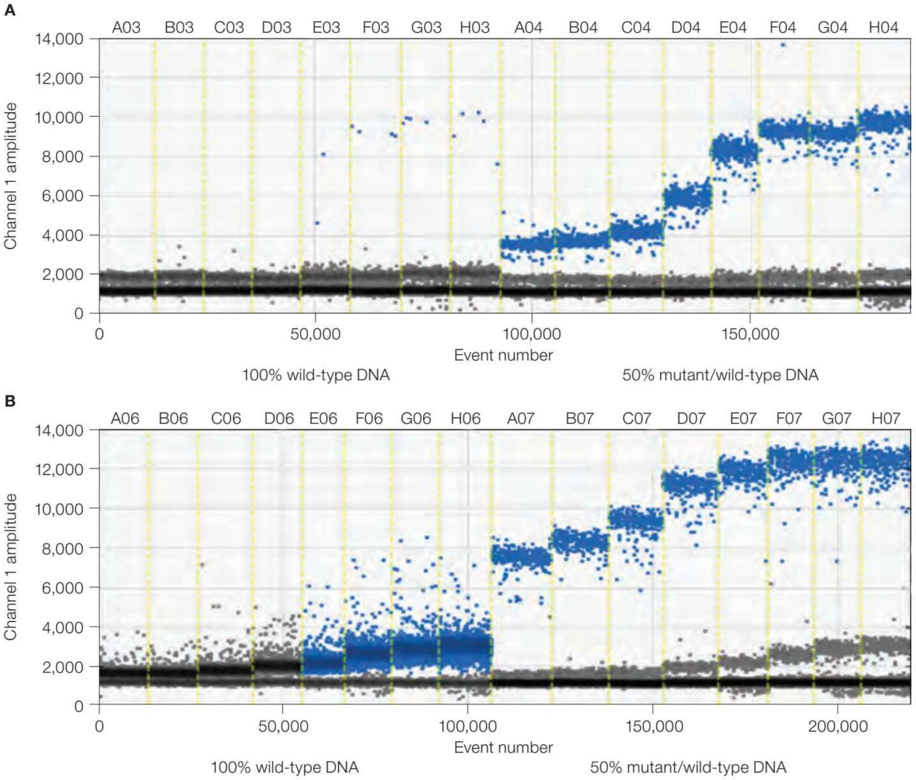


**Fig. 5.3. Two examples of SNP assay temperature gradients. A**, 1-D amplitude plot of the FAM-labeled mutant assay results of *PI3Kca_E545K*; **B**, 1-D amplitude plot of the FAM-labeled mutant assay results of *PI3Kca_H1047R*.

Once the best melting temperatures have been identified using 1-D plots, use 2-D amplitude plots to assess assay sensitivity. Look for no false positives or "spray" of droplets into the double-positive region in the 2-D amplitude plot of the wild-type–only samples.

The 2-D amplitude plot for the SNP assay should have no positive droplets in the double-positive region in the wild-type–only well. Wild-type–only control wells should have the same concentration of DNA as the highest concentration experimental well. Figure 5.4 shows an example of false positives or spray into the double-positive region. For more sensitive mutant detection, assay redesign may be recommended to resolve this.

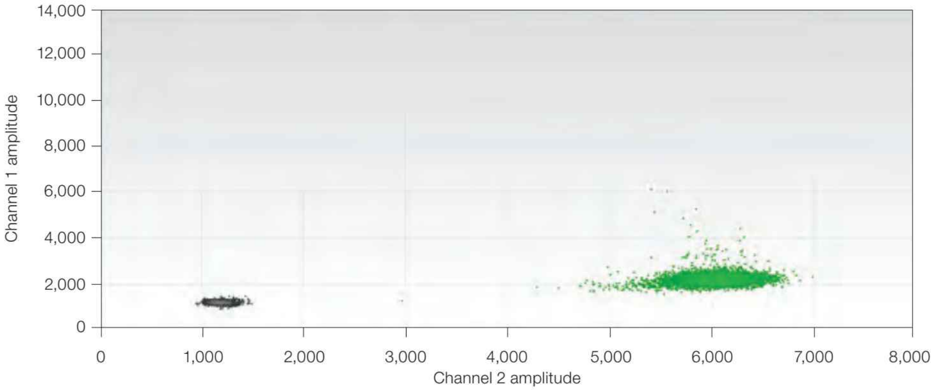


**Fig. 5.4. 2-D amplitude plot of a wild-type control well containing 2,000 copies/µl of an SNP assay with spray of false positives into the double-positive region of the plot.**

Figure 5.5 shows a 2-D plot of a single well with an assay for SNP *KRAS G12V* that indicates no false positives at 5,000 copies/µl. This assay can detect 0.001% mutant, or 1 mutant in 100,000 wild-type targets.

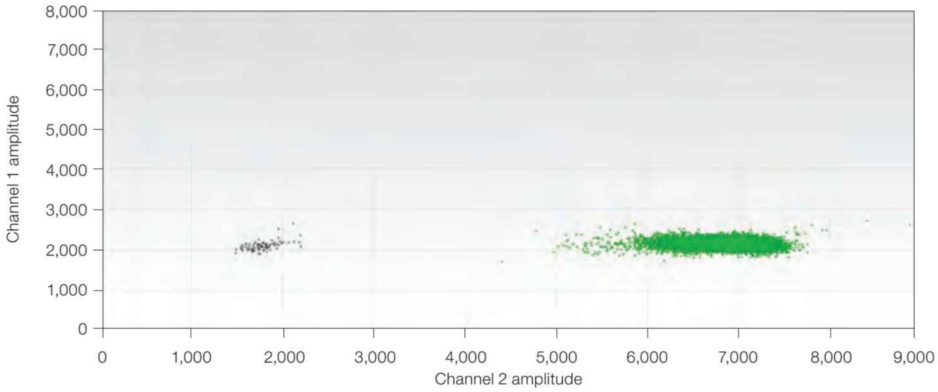


**Fig. 5.5. 2-D amplitude plot of a wild-type control well containing 5,000 copies/µl of an SNP assay with no spray of false positives into the double-positive region of the plot.**

Figure 5.6 shows a 2-D amplitude plot of four combined or merged wells of SNP *KRAS G12V* detecting 0.002% mutant.

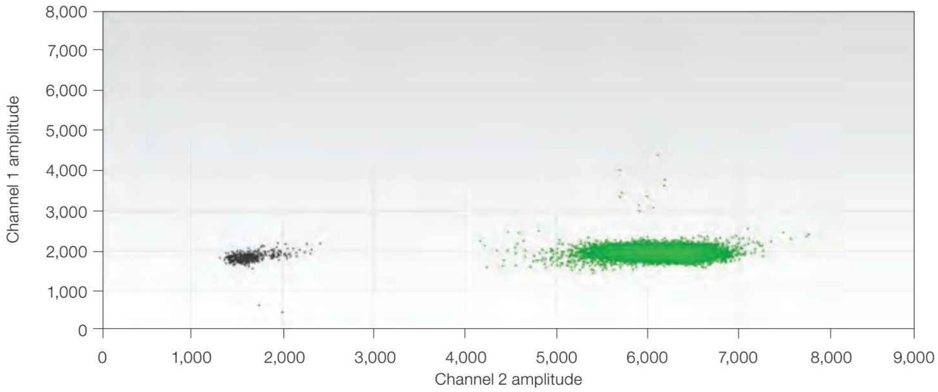


**Fig. 5.6. 2-D amplitude plot showing 0.002% mutant detection in four merged wells.**

## Interpreting 2-D Plot Results for SNP Assays

How does orthogonality affect assay performance? The shift of single-positive clusters toward the double-positive region makes it more difficult to separate single-positive droplets from double-positive droplets.

In Figure 5.7, the four clusters represent the droplets as follows:

- Blue cluster (top left), FAM-positive droplets with only mutant template
- Brown cluster (top right), double-positive droplets with both templates inside
- Gray cluster (bottom left), negative droplets with no template
- Green cluster (bottom right), VIC-positive droplets with only wild-type template

The orthogonality of the 2-D pattern means the four clusters are located spatially at right angles to each other. In an RMD assay, clusters are commonly deviated from the corner (right angles). For example, the blue cluster is shifted to right relative to the negative cluster in Figure 5.7, a result of wild-type probe (labeled with VIC) cross-reacting with mutant amplicon. Also, the double-positive cluster in an RMD assay is usually located between the two single-positive clusters rather than at the upper-right corner, due to cross-reactivity.

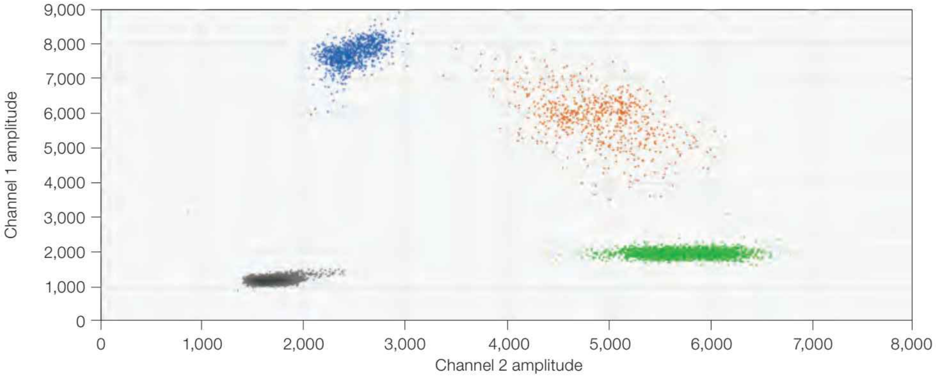


**Fig. 5.7. 2-D plot of *BRAF V600E* assay.**

## Statistical Considerations for Rare Detection Experimental Design

The limit of detection (LoD) is the minimum concentration of the mutant (rare) sequence that can be reliably differentiated from a negative control (100% wild type). In RMD, LoD is typically quoted as a ratio or a percentage: for example, 1 in 10,000, or 0.01%. In quantitative PCR, the LoD is largely a function of the cross-reactivity of the probes. In contrast, in ddPCR, the LoD is determined primarily by the number of wild-type molecules that are screened. The LoD can be adjusted to the precise requirement of any application by adjusting the number of wells, and therefore molecules, screened.

Consider a sample at a mutant or rare sequence concentration of 1 mutant or sequence in 10,000 wild type. To guarantee with 95% confidence that at least 1 mutant molecule will be screened in this sample, statistics dictates that at least 30,000 wild-type molecules must be screened, or three times the number of expected wild-type target molecules. This is about 100 ng/well of human DNA where the target has a copy of 1/haploid genome (Table 5.1).

# of molecules to screen = 3 × background molecules

Additional wells may be screened to ensure detection of more than one positive droplet. For ddPCR, the ability to merge multiple wells into a meta-well provides more experimental flexibility to tune the experiment to the required LoD. The number of cells or amount of DNA needed to screen a given number of background molecules is described in Table 5.1. The number of wells needed is a conservative estimate. The row in bold reflects the best performance in one well. Researchers may choose to screen additional wells to ensure detection of more than one positive droplet.

**Table 5.1. Requirements for different LoDs for RMD, assuming an ideal assay with a droplet false-positive rate of zero.**

| | Required Starting Material (human) | | | |
|---|---|---|---|---|
| **LoD** | **Total Copies to Screen** | **Diploid Cells** | **Amount of DNA** | **Number of Wells** |
| 1 in 1,000 | 3,000 | 1,500 | 0.010 µg | 1 |
| 1 in 10,000 | 30,000 | 15,000 | 0.10 µg | 1 |
| **1 in 25,000** | **75,000** | **37,500** | **0.25 µg** | **1** |
| 1 in 100,000 | 300,000 | 150,000 | 1.0 µg | 4 |
| 1 in 1,00,000 | 3,000,000 | 1,500,000 | 10.0 µg | 40 |

# Guidelines for Classifying Droplets in RMD Experiments

These guidelines assume proper experimental setup for rare mutation detection, including non-template control (NTC), negative control (e.g., wild-type–only DNA input at a level comparable to the highest expected test sample input), and positive control (e.g., enough mutant and wild-type DNA to create a double-positive cluster of greater than 100 droplets) wells. For a more complete discussion of experimental design, execution, and analysis, refer to Bio-Rad's Rare Mutation Detection Best Practices Guidelines (bulletin 6628).

## Guidelines for Droplet per Well Quality Control

For precise and accurate quantification, it is important to use only wells that have good droplet quality. The following guidelines will help you identify such wells.

- Select all wells containing the same assay and display droplets using a 1-D fluorescence amplitude plot (Figure 5.8)

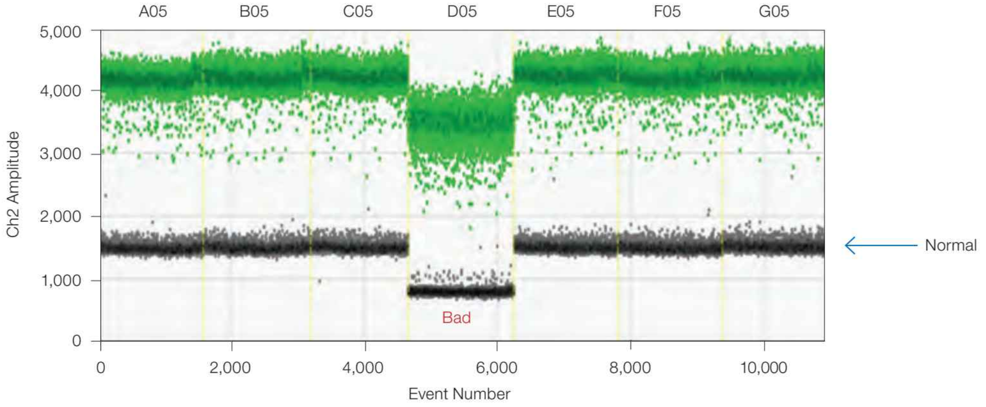


**Fig. 5.8. Assess wells for consistent droplet fluorescence amplitude.**

- Examine the double-negative droplet bands across all wells and reject any wells that have anamplitude significantly different from the norm (wells labeled "Bad" in Figure 5.8). This variance indicates poor droplet generation, most likely due to poor handling or mixing of samples, and is likely to generate erroneous concentration values
- Reject wells with fewer than 10,000 accepted events, as they may also provide aberrant quantification
- Inspect each well individually using a 2-D fluorescence amplitude plot to confirm acceptable droplet quality; 45° droplet spray should be minimal (Figure 5.9) and droplet clusters should be reasonably well defined
- Exercise caution in drawing conclusions from the test samples if positive or negative control wells are excluded for any reason

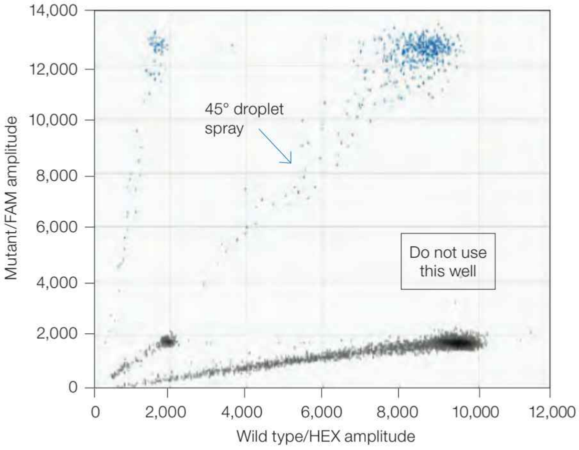


**Fig. 5.9. Identify and reject any wells with abnormal droplets.**

## Guidelines for Droplet Classification

The following guideline shows how to use NTC and positive control wells to achieve high specificity in your assay by eliminating false positive and false negative droplets.

- Select all wells in the 2-D droplet fluorescence amplitude view and classify the droplets into a maximum of four clusters (Figure 5.10). This can be achieved via auto analysis by using combined wells or manually by classifying the droplets using QuantaSoft™ Software's threshold or lasso function. QuantaSoft Software uses this classification to provide absolute concentration measurements of both mutant and wild-type targets, which are then used to arrive at the fractional abundance of the mutant. It is thus crucial that all clusters are properly classified
- Highlight all wells in the 2-D fluorescence amplitude plot and set the amplitude scale to "fixed" for easy subsequent comparison between individual wells
- Visualize the NTC wells to determine cleanliness of the reagents and workflow. In a clean reaction, there should be no positive droplets in NTC wells. More than the occasional mutant-positive droplet will impact the limit of detection and should be investigated to determine the root cause

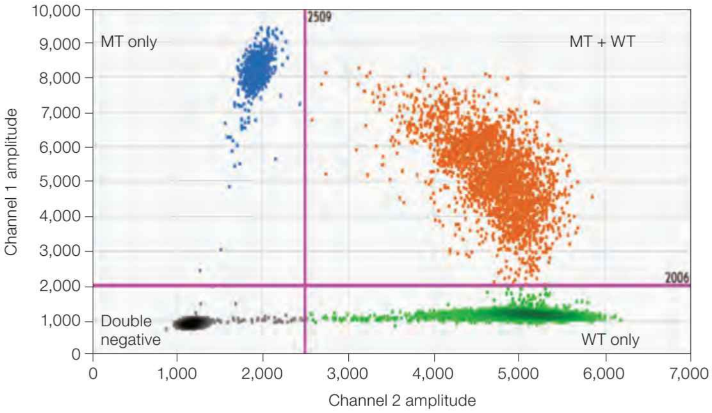


**Fig. 5.10. Classify droplets into clusters. WT, wild-type; MT, mutant.**

- Visualize NTC wells and reclassify mutant-positive droplets to negative to increase the specificity of your reactions. Note, however, that increased specificity comes at the risk of decreased sensitivity
- Visualize the positive control wells to ensure the reaction worked as desired. Inspect single-well 2-D droplet plots to confirm that the classification does not significantly miscategorize true mutant-positive droplets as wild-type–only or as double-negative droplets, which would result in false negatives. If you change a classification, return to the NTC wells to confirm that reclassification has not adversely affected the specificity of the assay
- If the controls do not perform acceptably, exercise caution in drawing conclusions from these data

## Sample Data Analysis

If all the above quality control metrics are satisfied, proceed to analyze the unknowns.

- Examine apparent mutant-positive wells. If there is not clear separation between the mutant-positive clusters, especially the double-positive cluster, and the mutant-negative clusters, especially the wild-type–only cluster, use the positive and negative controls to set a combined-well droplet classification that best separates the adjacent clusters. Apply it across all wells. Determine what is most critical to your needs and set the thresholds accordingly, sacrificing sensitivity in favor of specificity by minimizing false positives or sacrificing specificity in favor of sensitivity by minimizing false negatives (Figure 5.11). The impact of adjusting droplet classification can also be assessed by viewing the concentration values in the Results Table or Concentration chart

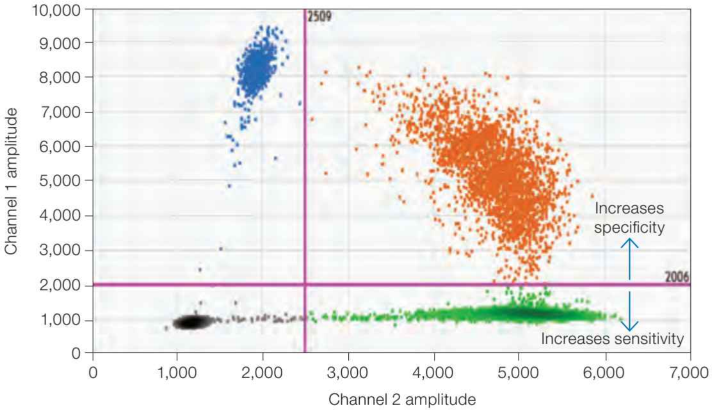


**Fig. 5.11. Adjust droplet classification to ensure clear separation between mutant positive and negative clusters.** Raising the FAM threshold line may increase specificity and decrease sensitivity. Lowering the threshold may result in the reverse.

For more detailed information, see Bio-Rad's Rare Mutation Detection Best Practices Guidelines (bulletin 6628).

# Recommended Controls

If a single wild-type or negative sample control well is run and the observed positive droplets are zero, then it is good practice to require at least three positive droplets in order to call a sample positive. The three positive droplets can be in either a single well or across merged wells. On the other hand, if you run a full plate of negative sample control wells and observe zero positive droplets, consider making a positive call based on a single positive droplet. However, we don't usually recommend this because it may be difficult to control for handling or contamination issues. If the same sample is spread across multiple wells, then the number of positive droplets should be pooled across all the wells and a similar number of negative control wells should be run.

Our guidelines assume that the number of positives in no template control (NTC) wells is also zero, which indicates good handling practices. If these numbers are not zero, the rate of potential false positives must be accounted for in calling a positive sample. Control wells may not be zero for the rare sequence. This could mean that contaminating template DNA is in the reaction wells. If positive droplets in the NTC wells occur, make sure that good laboratory practices for PCR are being followed in the laboratory (see Kwok and Higuchi 1989).

To limit workspace contamination, wipe down your pipets, tip boxes, and benchtops with 5–10% bleach, prepare master mixes in a template-free environment, add samples and generate droplets in an amplicon-free environment, read droplets in a room separate from the sample preparations, do not reuse DG8™ Droplet Generator Cartridges, oils, gaskets, plates, or pipet tips, and wear appropriate personal protective equipment that is discarded or confined to appropriate locations.

In addition to workspace contamination, consider careful experimental setup procedures before droplet generation. Be careful about anything that might produce aerosolized DNA. We recommend mixing your sample by pipetting up and down the full volume 10–12 times rather than vortexing or centrifuging your plate because removing the seal after these processes can spray contaminating DNA into other wells.

We recommend always running negative controls to monitor for all possible sources of false-positive droplets, given the risk of laboratory contamination when working with templates and amplicons. With an extremely sensitive technology such as ddPCR, we recommend running enough negative sample controls as part of every experiment so that you can demonstrate that the probability of calling a true negative sample incorrectly is below a certain threshold.

## Experimental Strategies for RMD

Once the number of target molecules to be screened has been determined for a given LoD, the number of wells required to screen that number of targets must be determined. In the QX100™ or QX200™ Droplet Digital PCR System, up to 1 µg of DNA can be loaded into a single well with a recommended limit of 5,000–6,000 copies/µl per well. To achieve a lower LoD than 1 in 25,000 using human DNA (with a copy number of one per haploid), more wild-type molecules can be screened by running the same sample in multiple wells.

## Factors that Impact RMD Calculations

Table 5.1 outlines RMD experimental design based on statistical requirements. Additional real-world factors will impact these numbers in an assay-specific and sample-specific way and should be considered when planning an RMD experiment.

- Sample availability — depending on the source (formalin-fixed, paraffin-embedded [FFPE] or fresh frozen), a typical tissue biopsy will provide <250 ng of sample, meaning <75,000 genome copies to screen and at best a 1 in 25,000 LoD. With a more typical 100 ng of DNA from a biopsy, an LoD of 1 in 10,000 is more practical

- Sample integrity and preparation — the calculations above assume that every target present can be successfully assayed and does not account for nonamplifiable DNA. Samples can have varying degrees of degradation and chemical damage. FFPE samples, for instance, are routinely used in cancer research, but can provide DNA of poor quality. Consider this impact when calculating the amount of starting material needed to effectively screen the desired number of target molecules

## Sample Preparation

To achieve optimal accuracy in copy number measurements, restriction digestion of genomic DNA is required. Restriction digestion separates tandem gene copies, ensuring proper random partitioning into droplets. Restriction digestion can also reduce sample viscosity and improve assay performance by improving template accessibility.

# Rare Sequence Detection

An RSD application might require one or two assays. If the goal is to quantify the amount of target sequence in a given volume of starting material, a single assay is used (Figure 5.12). If the goal is to quantify the amount of target sequence with respect to a given reference sequence, two independent assays are required (Figure 5.13). In either case, assay design and optimization for RSD has less risk of cross-reactivity of the target assay with background DNA.

## RSD Experimental Strategies

Some RSD applications require reliable quantification of rare sequences while others require detection of a rare sequence. This difference dictates the lower bounds of sensitivity for a given assay and application. For RSD, the LoD can be defined either in terms of the total volume of material analyzed or in terms of the number of copies of some type of background DNA.

In RSD, absolute quantification of the target sequence is often required. In general, ddPCR can provide an accurate quantification of a rare target sequence at a low concentration. This eliminates the need for absolute standards and standard curves and improves reproducibility across experiments and laboratories. In addition, in ddPCR, small variations in PCR efficiency between wells have no effect on the measured concentration.

When absolute quantification is applied to an RSD application, an important consideration is the limit of quantification, or LoQ, which is the lowest concentration you can reliably measure within a predetermined variance, or coefficient of variation (CV). For example, if you want to quantify within 20%, the LoQ is the lowest concentration with which you can reliably measure to within ±20% of the real value. For RSD, to reliably detect 1 in 100,000 cells, at least 300,000 background cells must be screened. Similarly, to achieve an LoD of 1 in 1 ml of sample, 3 ml of sample must be screened.

Two different experimental setups are recommended for RSD applications, depending on whether detection is with respect to a starting volume of sample or with respect to a second background target that requires quantification.

## Case 1: Quantification with Respect to Total Starting Volume

To detect foreign DNA in 5 ml of lake water, 15 ml of lake water must be screened. The experiment is shown in Figure 5.12 for the case of a single ddPCR well. Depending on how much total DNA is extracted from the 15 ml sample, more than one well may be needed to analyze the sample because up to 2.5 µg of total DNA can be loaded into one reaction well.

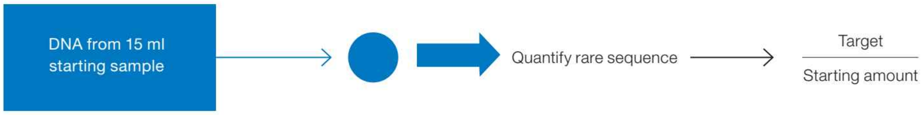


**Fig. 5.12. Strategy for detecting rare foreign DNA in a sample of defined volume.**

## Case 2: Quantification with Respect to Second DNA Sequence

To detect a very low target concentration in a high background sample, for example 1 copy of virus/100,000 peripheral blood mononuclear cells (PBMCs), the sample can be analyzed at two different concentrations (Figure 5.13). The high-concentration wells provide sensitive detection of the rare sequence while the low-concentration wells enable quantification of the background sequence. For example, to reliably detect an average of 1 virus in 100,000 PBMCs, we must screen 300,000 PBMCs, or 2 µg of DNA (Table 5.2).

This requires three wells on the QX100 System: two wells at high concentration to screen enough PBMCs for the presence of rare virus, and a third well at a lower concentration to quantify the PBMCs. This experimental setup is shown in Figure 5.13.

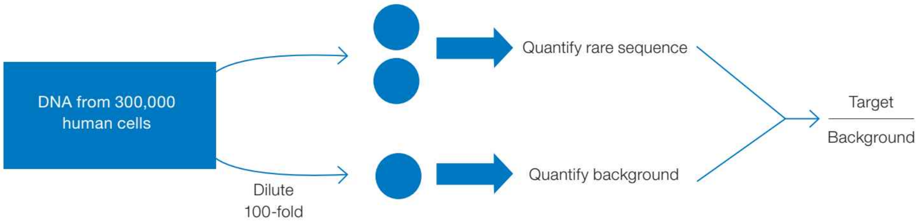


Fig. 5.13. Detection of a very low target concentration in a high background (such as a low viral load in a given number of PBMCs) by analyzing samples at two different concentrations.

The target sequence (the "rare" sequence) might be quantified at either concentration, depending on the amount present in the starting sample. The background sequence is quantified in the low concentration well.

In the examples shown in Table 5.2, the LoD is defined with respect to the number of human cells. The number of wells needed is a conservative estimate that considers that droplets can be lost due to workflow or quality control specifications.

**Table 5.2. Case 2: Experimental setup for different LoD and LoQ in rare sequence detection.**

| | | | | Number of Wells | |
|---|---|---|---|---|---|
| LoD | LoQ (<10% CV) | Human Diploid Cells | Amount of DNA, µg | High Concentration | Low Concentration |
| 1 in 5,000 cells | 100 in 15,000 cells | 15,000 | 0.10 | 1 | 0 |
| 1 in 12,500 cells | 100 in 37,500 cells | 37,500 | 0.25 | 1 | 0 |
| 1 in 100,000 cells | 100 in 300,000 cells | 300,000 | 2.0 | 2 | 1 |
| 1 in 500,000 cells | 100 in 1,500,000 cells | 1,500,000 | 10.0 | 10 | 1 |

In the example given, the measurement error (CV) is introduced at the time of subsampling (at the time of the blood draw), not by the measurement system. Poisson statistics dictate that if *N* target molecules are measured in a sample, the standard deviation of the measurement is roughly the square root of *N*. In this example, if 100 copies of virus are present in the sample, the standard deviation of the measurement is 10 and the CV is 10/100 = 10%. This means that we can accurately quantify, to ±10%, 100 or more copies of virus in 100,000 PBMCs. No detection system can do better than this theoretical limit.

## Factors that Impact RSD Calculations

- False-positive rate — the false-positive rate must be measured and considered when designing experiments to achieve very low LoD or accurate quantification
- Sample integrity and preparation — it may be useful to spike a PCR control into the original sample and use a second assay in the same well to measure its level. This enables you to control for PCR inhibitor and sample preparation failures
- Pipetting accuracy in dilutions — errors introduced by pipetting may contribute to the overall accuracy of the measurement

# Liquid Biopsy

Liquid biopsy is the sampling and evaluation of circulating cell-free DNA (cfDNA) and is an innovative, noninvasive strategy for detecting and monitoring disease. It enables low-risk, low-cost diagnostics that may facilitate effective and early treatment decisions by offering real-time mutation profiling and sensitive and specific detection and quantification of cfDNA and circulating tumor DNA (ctDNA) from biofluids including plasma, urine, and CSF (cerebrospinal fluid). Early detection of ctDNA may result in earlier administration of a targeted therapy specific for the patient's mutation signature, thereby improving chances of the treatment's effectiveness. Furthermore, treatment strategies can be adjusted by monitoring resistance, recurrence, and relapse of the disease based on quantification of ctDNA and the emergence of any new mutation biomarkers.

Accurately quantifying cfDNA or ctDNA is often challenging, as quantities are typically very low and highly variable, depending on cancer type and disease stage. In many cases, especially in early, localized disease, 1 ml of plasma will yield only 10 ng of cfDNA or less. And from this, only a small fraction will be ctDNA containing the genetic signatures of the tumor. Most cfDNA will be derived from normal, healthy cells, which increases wild-type DNA background and results in mutation frequencies of <1%. Thus, a highly sensitive method is required to detect and accurately quantify these low-level mutations.

## Mutation Detection in Cell-Free Plasma Samples

Droplet Digital™ PCR (ddPCR™) is a sensitive and inexpensive technology for absolute quantification of cfDNA and for mutation detection in ctDNA. As shown in Figure 5.14, Bio-Rad's ddPCR *KRAS* Screening Multiplex Kit allows sensitive and precise detection down to a fractional abundance of 0.2% for multiple mutations in cell-free plasma DNA samples from cancer patients. For more detailed information, see Bio-Rad bulletin 6679.

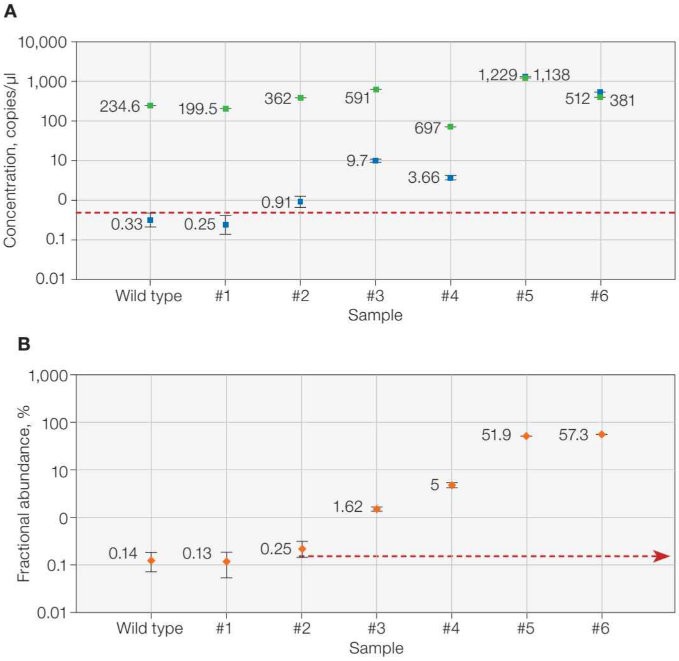


**Fig. 5.14. Screening cell-free plasma DNA samples using Bio-Rad's ddPCR *KRAS* Screening Multiplex Kit. A**, concentration plot and **B**, fractional abundance plot (percentage mutant) of wild-type *KRAS* (🟩), mutant *KRAS* (🟦), and percentage mutant (🟧) from six MDACC celll-free plasma DNA samples, demonstrating the range of percentage mutant detection.

When using cell-free plasma samples, at least 5 ng of amplifiable DNA (~1,500 copies) per sample is required to reliably detect mutations present at 0.2% (Figure 5.15), depending on false positive background, sample quality, and amount of amplifiable DNA.

One of the main challenges of isolating cfDNA is minimizing contamination by wild-type DNA from normal cells, which may reduce the mutant signal present from ctDNA. Recently, studies have shown that collection of blood into tubes treated with cell stabilization agents such as Cell-Free DNA BCT Tubes (Streck) may help resolve this issue by preventing lysis of blood cells post-collection. Additionally, two-step centrifugation protocols can be employed to remove blood and other contaminating cells, including platelets, which are the major contributors of DNA in plasma cfDNA, and therefore lead to high wild-type background.

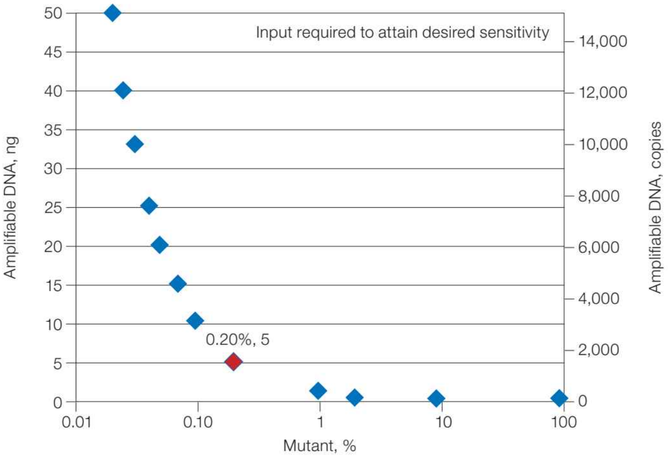


**Fig. 5.15. Cell-free plasma samples yield highly variable amounts of amplifiable DNA.**

## Rare Mutation Detection in Formalin-Fixed, Paraffin-Embedded (FFPE) Samples

DNA quantification from FFPE samples is widely used because it is an inexpensive and established procedure. However, formalin fixation often causes DNA damage, including DNA-DNA crosslinks, DNA-protein crosslinks, abasic sites, oxidized bases, and deaminated cytosines. Additionally, FFPE DNA has very low yields after purification and <10% of this DNA is amplifiable.

As shown in Figure 5.16 pretreatment of an FFPE sample with uracil DNA glycosylase (UDG) can digest deaminated cytosines (C>T), reducing false-positive signals.

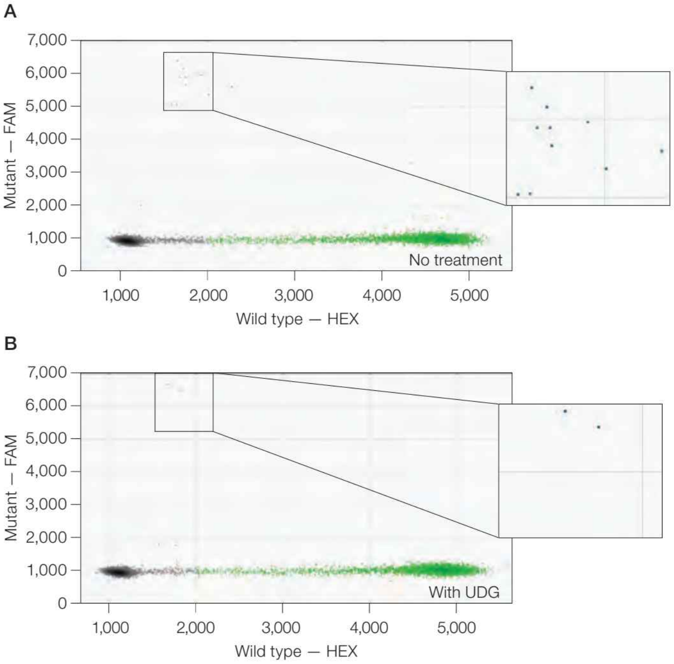


**Fig. 5.16. Effect of treatment of FFPE samples with UDG.** When pretreated with UDG, an FFPE sample (M2W, grossly normal) showed significantly lower G13D mutant positive droplets (**A** vs. **B**, 12 vs. 2 events, respectively), altering the sample call from mutant to wild type.

Furthermore, loading less sample allows better DNA amplification when using FFPE samples that include PCR inhibitors, as can be seen in Figure 5.17.

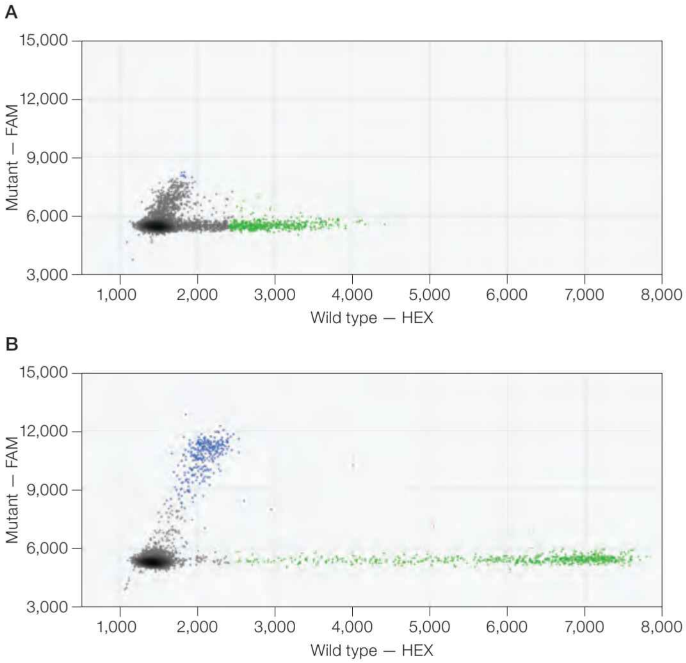


**Fig. 5.17. Effect of loading less FFPE sample. A**, when loading 5 µl, 20% of sample is amplifiable, but any inhibitors that are present impact positive amplitudes. **B**, reducing sample amount to 2 µl allows better amplification.

In addition to these considerations, formalin fixation should be processed as soon as possible and all controls should be generated from similar samples, as sample type can influence the limit of detection (LOD).

# References

Benlloch S et al. (2006). Detection of BRAF V600E mutation in colorectal cancer: Comparison of automatic sequencing and real-time chemistry methodology. J Mol Diagn 8, 540–543.

Kwok S and Higuchi R (1989). Avoiding false positives with PCR. Nature 339, 237–238.

Scott LM (2011). The JAK2 exon 12 mutations: A comprehensive review. Am J Hematol 86, 668–676.

Whitehall V et al. (2009). A multicenter blinded study to evaluate KRAS mutation testing methodologies in the clinical setting. J Mol Diagn 11, 543–552.

# 6 Gene Expression

## Overview

Reverse transcription quantitative PCR (RT-qPCR) is a commonly used method in gene expression studies. It is straightforward, sensitive, and has a wide dynamic range. There are two types of approaches for the RT-qPCR reaction: one-step and two-step RT-qPCR.

### One-Step RT-qPCR

One-step RT-qPCR simplifies the reaction setup by combining the first-strand cDNA synthesis (reverse transcription) and qPCR in one mixture. It also greatly reduces the possibility of contamination by eliminating the cDNA-to-PCR operation step. One-step RT-qPCR can use only a limited number of probes per sample, but because it amplifies the whole sample, the sensitivity is greatly enhanced. The disadvantage of one-step RT-qPCR is that it is less amenable to multiplex assays and allows for less flexibility in priming strategies.

### Two-Step RT-qPCR

Two-step RT-qPCR performs the first-strand cDNA synthesis reverse transcription and qPCR in separate mixtures. This method allows for the measurement of multiple messages from a single RNA sample. It also enables you to use different PCR reaction conditions and priming methods.

To measure gene expression with PCR, you must first convert the RNA into DNA by reverse transcription. There are three types of primers that may be used in reverse transcription:

- Oligo(dT) — priming with oligo(dT) results in cDNA synthesis that is biased to the 3' end of polyadenylated transcripts and will create only cDNA from mRNA templates
- Random primers — random priming is not subject to end bias and is not limited to mRNA, but it is sensitive to the sequence and secondary structures of the template
- Sequence-specific primers — sequence-specific primers can be designed adjacent to the PCR target, but the ability to perform multiplex qPCR from an individual sample is limited

Similar to RT-qPCR, reverse transcription Droplet Digital™ PCR (RT-ddPCR) approaches can also use a one-step or two-step protocol. Choose your approach based on your experimental purpose.

## Two-Step Reverse Transcription ddPCR

### Obtain RNA

Use a commercial kit to extract RNA. Store at 100 ng/µl in 1/10 TE buffer (0.1x TE) or other appropriate buffer at –80°C.

### Generate cDNA

Generate cDNA according to standard protocols. We recommend Bio-Rad's iScript™ Select cDNA Synthesis Kit for oligo(dT) or gene-specific priming. For random priming, we recommend the iScript Advanced cDNA Synthesis Kit for RT-qPCR. Follow the instructions in the manual of the respective cDNA kit.

Once the reverse transcription is complete, reduce the concentration of cDNA to about 0.2 ng/µl RNA equivalent and use 5 µl per Droplet Digital PCR (ddPCR™) reaction (total volume 20 µl). Typically 1 ng (5 µl of 0.2 ng/µl) of RNA-equivalent cDNA per ddPCR reaction is adequate to measure most of the transcripts reliably. If the RNA is highly degraded or very low quality, as is the case with RNA from formalin-fixed, paraffin-embedded (FFPE) samples, or the transcript of interest is expected to be found at less than 1 copy/cell, such as a transcript from a cancer cell in a large background of normal cells, the previous guideline of 1 ng/reaction no longer holds. Under these situations, up to 10 µl of the RT reaction can be added to one ddPCR reaction without altering its performance.

## One-Step RT-ddPCR Advanced Kit for Probes

The One-Step RT-ddPCR Advanced Kit for Probes follows the same workflow as the ddPCR supermix for probes, with the benefit that you can now directly partition sample RNA instead of DNA. The sample is partitioned into 20,000 droplets, with target and background RNA randomly distributed among the droplets. An RNase inhibitor included in the formulation minimizes template degradation during reaction setup and droplet generation. After reverse transcription, the resulting cDNA is amplified for target detection using TaqMan hydrolysis probes. After PCR amplification, each droplet provides a fluorescent positive or negative signal indicating the target RNA was present or not present after partitioning. Each droplet provides an independent digital measurement. Positive and negative droplets are counted and software calculates the concentration of target RNA as copies/µl.

The One-Step RT-ddPCR Advanced Kit for Probes is formulated for efficient and sensitive reverse transcription over a wide linear dynamic range of input RNA for ddPCR. Use a commercial kit to extract RNA, then store RNA at 100 ng/µl in 1/10 TE buffer (0.1x TE) or other appropriate buffer at –80˚C. A unique hot-start reverse transcriptase enables convenient reaction setup. The reverse transcription reaction is performed at 55–60°C, enhancing the specificity and efficiency of primer-mediated cDNA conversion. The thermostable enzymes allow RNA template to be reverse transcribed and subsequently amplified in the same reaction tube. Template-specific primer annealing at elevated temperatures significantly improves stringency and melting of secondary structures.

## Data Analysis

In QuantaSoft™ Software, create a plate map by designing a new plate with a layout according to your experimental design. Designate the experiment type as Absolute Quantification (ABS) in the Well Editor window during plate setup. Designate which assay is the target and which is the reference assay (Channel 1 or 2) in the Well Editor window.

If the plate was set up for ABS analysis, automatic thresholding will determine concentrations and populate the data tables in the analysis mode of the software. To get the ratio of target over reference for gene expression, a duplex assay must be run with two different probes (FAM and HEX [or VIC]).

In QuantaSoft data analysis, a button labeled Ratio will become active if the experiment contains a duplex assay set up with a FAM assay and a HEX or VIC assay (Figure 6.1). Clicking on the **Ratio** button plots the relative ratio of a/b (the concentration of the FAM assay over the concentration of HEX or VIC assay). You can also click the **Inverse** button (Figure 6.1) if you switched the assay dyes where FAM is the reference and HEX or VIC is the target (the inverse makes the ratio b/a instead of a/b).

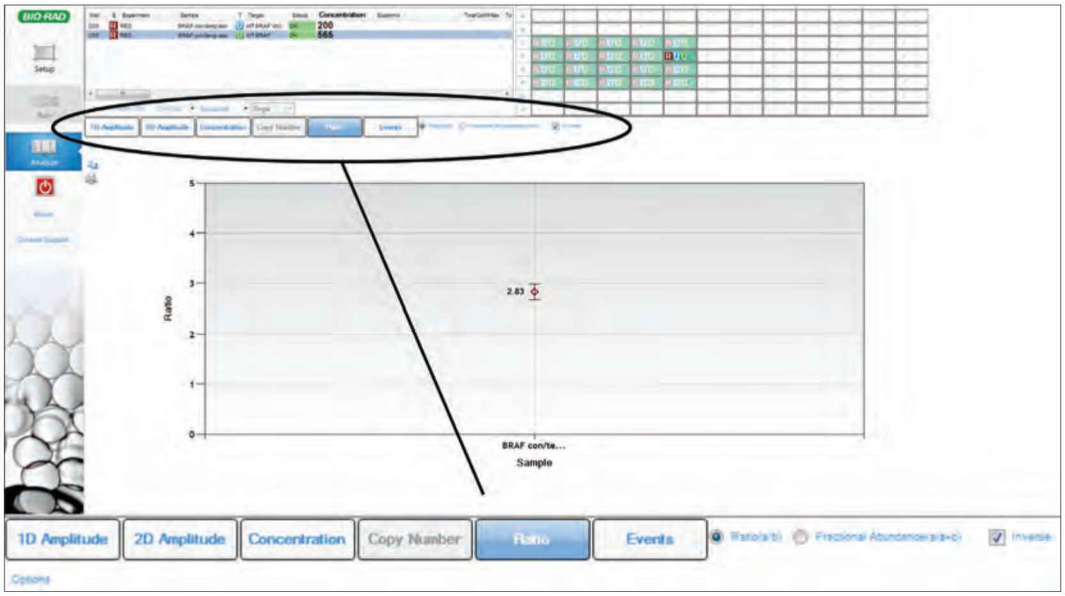


Fig. 6.1. QuantaSoft Software ratio feature and inverse checkbox for gene expression ratios.

## ddPCR Gene Expression Data

As shown in Figure 6.2, cDNA copies were made on three in vitro transcribed RNAs with the One-Step RT-ddPCR Kit for Probes or Two-Step RT-qPCR with iScript Advanced cDNA Synthesis Kit for RT-qPCR. The data are generally comparable on most of the assay locations. A few sites are much lower in one-step RT-ddPCR because those sites may be involved in secondary structures and the priming sites might not be accessible to the reverse transcription enzyme. You must decide whether one-step or two-step RT-ddPCR is best for your experiment.

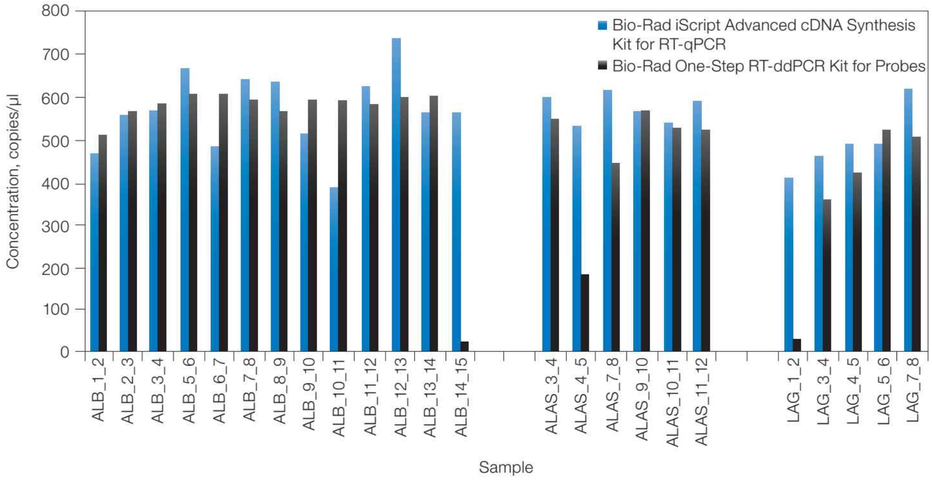


**Fig. 6.2. Comparison of one-step RT-ddPCR and two-step RT-ddPCR with in vitro transcribed RNAs spiked in Ambion human brain reference RNA.**

# *HER2* Study

The human epidermal growth factor receptor 2 gene (*HER2*) is encoded by the *ERBB2* proto-oncogene and is involved in signal transduction for cell growth and differentiation. It is a cell surface receptor for tyrosine kinase and a proto-oncogene. Overexpression of *ERBB2* is of clinical relevance in breast cancer because of its prognostic value in correlating elevated expression with worsening clinical outcome. *ERBB2* assessment is also important because successful antitumor treatment with Herceptin is strongly correlated with *ERBB2* overexpression in the tumor (about 30% of all breast tumors overexpress *ERBB2*).

## Data Analysis Results

Results demonstrate that ddPCR can be used to assess expression levels of *HER2* transcripts in FFPE human breast tissues samples that were concurrently assessed via pathologists using fluorescence in situ hybridization (FISH) and immunohistochemistry (IHC) methods.

Figure 6.3 illustrates the determination of *ERBB2* transcript levels relative to the *EEF2* reference gene. The “best coverage” *ERBB2* assay was tested with four fresh-frozen breast tumor RNA samples (OriGene Technologies, Inc). FAM (*ERBB2*) concentration, VIC (*EEF2*) concentration, and normalized *ERBB2* to *EEF2* concentrations are shown in Figure 6.3 (blue, green, and maroon, respectively).

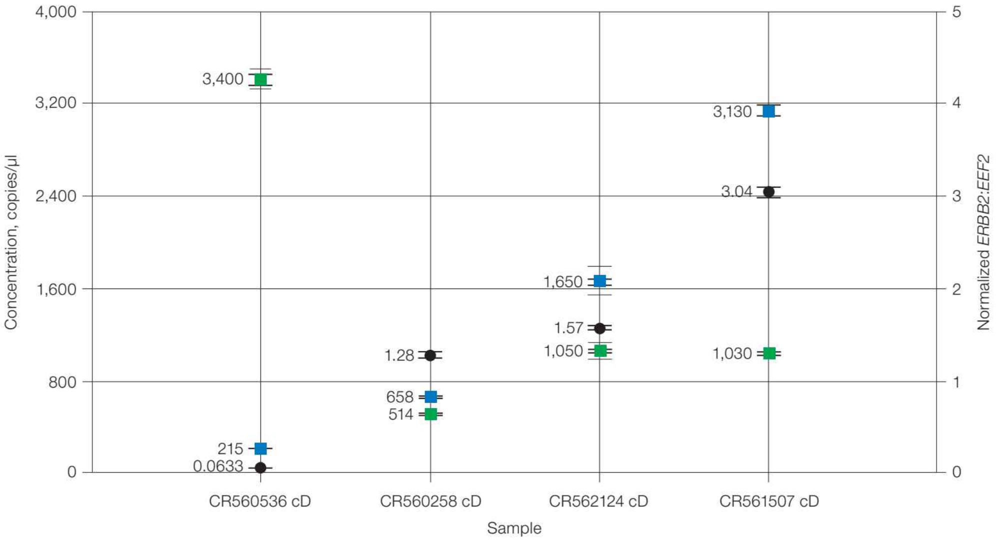


**Fig. 6.3. Concentrations of *ERBB2* and *EEF2* gene expression.**

Figure 6.4 shows a twofold change in sample concentration that demonstrates linearity of the assay spanning two log orders.

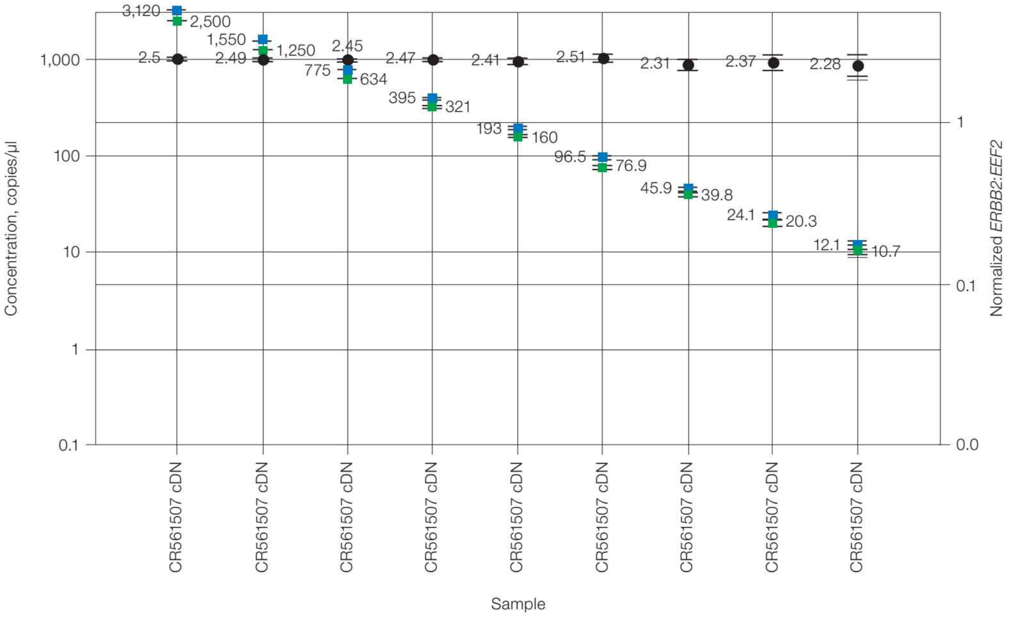


**Fig. 6.4. Twofold dilution series demonstrating linearity across two log orders.**

Figure 6.5 is an example of normal breast tissue compared with *HER2*+ tissues (fresh frozen).

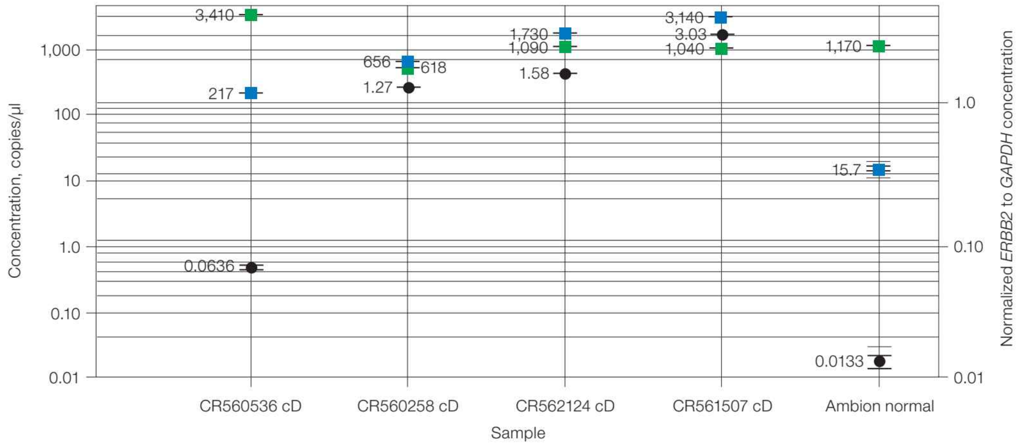


**Fig. 6.5. Comparison of *HER2* expression levels of fresh frozen tumor versus normal tissue.**

Figure 6.6 shows the quantification of RNA transcript levels compared with DNA copy number. Commercial (Applied Biosystems) *ERBB2*, *GAPDH*-PL, and *EEF2* gene expression TaqMan assays were tested with breast tumor FFPE samples. ddPCR was used to determine the amounts of transcripts in a subset of 12 samples (from the genomic copy number study above) that are normalized to commonly expressed genes *GAPDH* and *EEF2*.

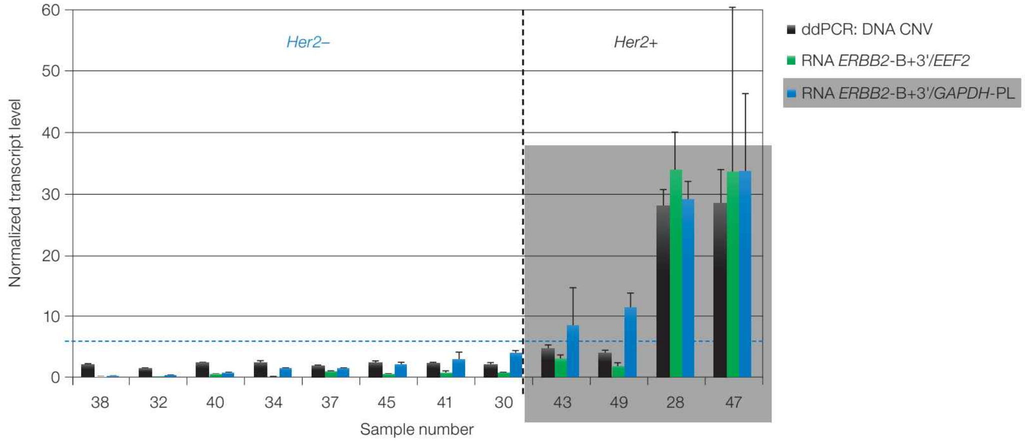


**Fig. 6.6. Rank ordering of gene expression levels in 12 clinical samples and genomic copy number status of *ERBB2*.**

When we studied the clinical FFPE samples using ddPCR and standard FISH and IHC methods, our results demonstrated that ddPCR can rank order the samples in complete agreement with the current standard methods and that ddPCR has the added benefit of providing quantitative results.

Table 6.1 shows a comparison of IHC, FISH, genomic copy number, and transcript level in clinical samples. The table shows the capability of ddPCR to determine gene copies and *ERBB2* cDNA transcript levels when normalized to a reference transcript (*EEF2* or *GAPDH)*. ddPCR results are in excellent agreement with pathology results, and have the added benefit of being quantitative.

**Table 6.1. Comparison of clinical pathology results with ddPCR gene expression data.**

| | | **Clinical Pathology** | | **ddPCR: CNVs or Normalized RNA Levels** | | |
|---|---|---|---|---|---|---|
| **Sample Number** | **Year Specimen Taken** | ***HER2* IHC Score** | **FISH *HER2*/*CEP17* Ratio** | **DNA *HER2*/*CEP17*** | **RNA *ERRB2*-B+3'/*EEF2*** | **RNA *ERRB2*-B+3'/*GAPDH*** |
| 38 | 2009 | 1+ | 0.8 | 2.20 | 0.22 | 0.27 |
| 32 | 2010 | 1+ | 1.2 | 1.56 | 0.29 | 0.46 |
| 40 | 2008 | 2+ | Negative | 2.46 | 0.54 | 0.88 |
| 34 | 2009 | 1+ | Not done | 2.61 | 0.17 | 1.42 |
| 37 | 2008 | 0 | Not done | 1.96 | 0.98 | 1.54 |
| 45 | 2006 | Negative | Not done | 2.55 | 0.63 | 1.89 |
| 41 | 2008 | 1+ (resection) | Negative | 2.53 | 0.85 | 3.01 |
| 30 | 2009 | 1+ | Not done | 2.37 | 0.86 | 3.87 |
| 43 | 2007 | 2+ | Positive | 4.76 | 2.97 | 8.31 |
| 49 | 2006 | 3+ | Not done | 4.15 | 1.79 | 11.3 |
| 28 | 2010 | 3+ | Not done | 28.0 | 33.9 | 29.1 |
| 47 | 2006 | 3+ | Not done | 28.4 | 33.6 | 33.7 |

CNV, copy number variation; FISH, fluorescence in situ hybridization; IHC, immunohistochemistry.

ddPCR provides the advantage of accurate and precise measurements of transcript levels. It also provides accurate genomic copy number determinations of samples with low- and high-order copy changes, determined by the ratio of the target concentration to the reference concentration. Important considerations for ddPCR applied to gene expression studies include the type of method used to obtain cDNA, one-step versus two-step reverse transcription, and optimizing assays for maximum performance.

# 7

# ddPCR™ Genome Edit Detection Assays

ddPCR Genome Detection Assays offer a fast, precise, simple, and cost-effective method for detection of genome editing events. Assays can be obtained for any target using Bio-Rad's easy-to-use, intuitive Digital Assay Site.

ddPCR Genome Edit Detection Assays:

- Enable rapid assessment of HDR and NHEJ edits generated by CRISPR-Cas9 or other genome editing tools
- Detect editing events present at frequencies of ≤0.5%
- Provide absolute quantification of genome editing events from as little as 5 ng of total gDNA (cells from single well of a 96 well plate)
- Distinguish between homozygous and heterozygous edits in clonal populations
- Results are easily analyzed using QuantaSoft™ Analysis Pro Software

## Genome Editing

Genome editing with site-specific, programmable nucleases has revolutionized the ability to edit the genome of any cell. Nucleases, including zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and clustered regularly interspaced short palindromic repeat (CRISPR)–associated Cas nucleases, produce site-specific DNA double-strand breaks (DSBs). DSBs can be repaired by imprecise, error-prone non-homologous end joining (NHEJ) or by homology directed repair (HDR) pathways, leading to targeted mutagenesis (Figure 7.1).

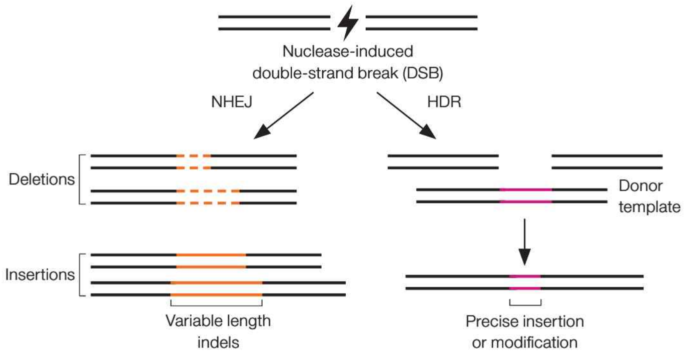
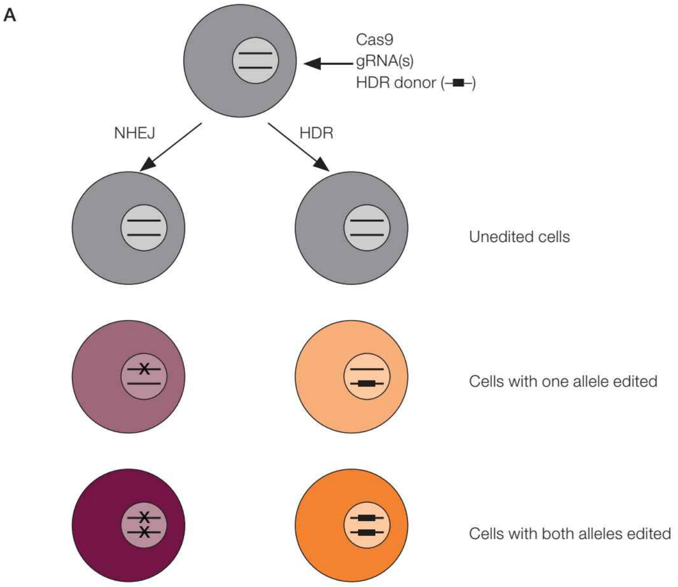
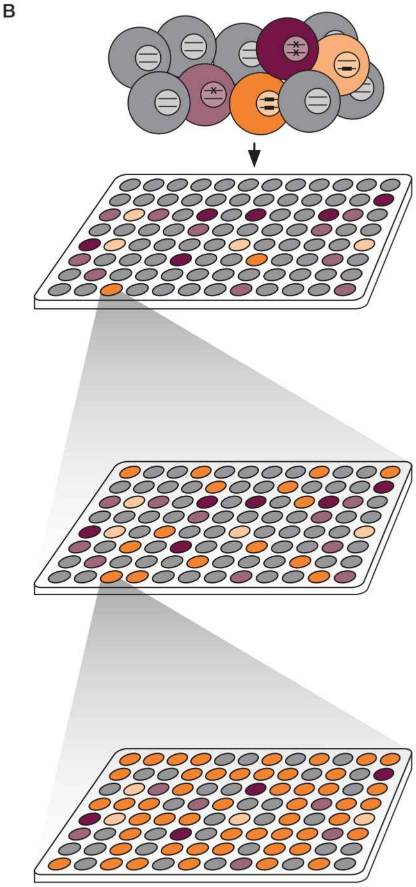

Nuclease-induced
double-strand break (DSB)
NHEJ
HDR
Deletions
Insertions
Donor
template
Variable length
indels
Precise insertion
or modification

**Fig. 7.1. Targeted genome editing.** DSBs introduced by nucleases can be repaired by non-homologous end joining (NHEJ) or homology-directed repair (HDR). NHEJ produces insertion and/or deletions (indels) of variable length at the site of the DSB. HDR can introduce precise point mutations or insertions using a DNA donor template.

# Creation of Recombinant Cell Lines using CRISPR-Cas9

Introducing CRISPR-Cas9 and guide RNA (gRNA) into cells (with or without a donor template) results in DSBs, which the cellular machinery repairs by NHEJ or HDR. This results in a mixed population of cells with heterogeneous indel errors and varying allelic editing frequencies (Figure 7.2A). Transfected cells are harvested and analyzed 24–72 hours post-transfection to measure genome editing frequency at the desired locus. Clonal cell lines can then be generated using serial dilutions to isolate single cells followed by an expansion period. Each clonal cell line is then assayed to verify the genome editing event (Figure 7.2B).


A
Cas9
gRNA(s)
HDR donor (–■–)
NHEJ
HDR
Unedited cells
Cells with one allele edited
Cells with both alleles edited



Use Droplet Digital PCR to:

- Quickly assess genome edit efficiency
- Evaluate guide RNAs, transfection reagents, and other experimental conditions
- Select pools for cell line creation

- Detect genome edits present at low frequencies from cells from a single well
- Accelerate cell line development by avoiding expansion of cell populations required to obtain large amounts of DNA

- Distinguish between homozygous and heterozygous clones

**Fig. 7.2. Creation of clonal cell lines containing desired genome edits. A**, CRISPR-mediated genome editing can result in unedited cells, cells with one allele edited, and cells with both alleles edited. **B**, To isolate cells with both alleles altered by HDR, a mixed pool of cells is serially diluted. Subpopulations are screened using Droplet Digital PCR (ddPCR), wells with cells of interest are selected and further subdivided until a clonal population containing the desired edit is obtained.

## Droplet Digital PCR (ddPCR) for Ultra-Sensitive Quantification of Genome Editing Events

The efficiency and mutation spectrum generated by genome editing varies substantially depending on the type of cells used, transfection method, target sequence, and many other factors. Therefore a simple, quick method is required to accurately characterize and quantify genome-editing events.

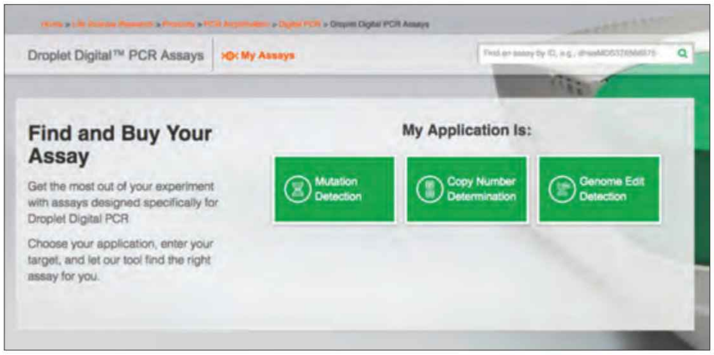
The T7 Endonuclease 1 or Surveyor assays are commonly used for detecting genome editing events. However, these methods are semi-quantitative, offer limited sensitivity, are prone to false-positives, and suffer from high background signal when sequence polymorphisms are present. These assays require a large amount of starting material necessitating significant cellular expansion. Further, these methods have limited utility for screening of single-cell derived clones. For a typical diploid target locus, a clone with both alleles successfully altered via genome editing, will be indistinguishable from a clone with one mutated allele and one wild-type allele.

Next generation sequencing is a comprehensive method for assessing genome editing but the turn around time is several weeks, analysis is complex, and costs are high. This makes this method unsuitable for routine screening.

Droplet Digital PCR offers a superior ultrasensitive, rapid, and low-cost method for absolute quantification of genome editing events. ddPCR allows absolute quantification from very small amounts of starting material. Clones harboring homozygous and heterozygous edits can be distinguished and ddPCR Genome Edit Detection Assays can be easily designed for any target of interest. This enables a much more rapid and efficient workflow and provides the ability to quickly identify and focus on desired clones.

## ddPCR Genome Edit Detection Assays

Genome edit detection assays, optimized specifically for ddPCR, can be ordered instantly for any target of interest on Bio-Rad's easy-to-use Digital Assay Site (Figure 7.3). Bio-Rad's assay design algorithms take into consideration everything from primer/probe placement and melting temperature to GC content and even genome specificity to design ddPCR Genome Edit Detection Assays with superior sensitivity, specificity, and accuracy, that accelerate your time to results.



Find and Buy Your Assay

Get the most out of your experiment with assays designed specifically for Droplet Digital PCR

Choose your application, enter your target, and let our tool find the right assay for you.

**Fig. 7.3. Order ddPCR Genome Edit Detection Assays for any target on the Digital Assay Site.** Easy, step by step workflow allows design of NHEJ and HDR Genome Edit Detection assays in minutes.

## Detection of HDR Edits using ddPCR HDR Genome Edit Detection Assays

ddPCR Homology Directed Repair Genome Edit Detection (HDR GED) Assays use a modified mutation detection assay strategy. The ddPCR HDR GED Assay contains primers, a FAM probe that is designed to bind the HDR edit, and a non-fluorescent probe that prevents binding of the FAM probe to unedited alleles. ddPCR HDR GED Assays must be run duplexed with a reference assay containing a HEX probe. Thus, two amplicons are generated (Figure 7.4). FAM positive droplets represent the population of alleles with the desired HDR edit and the HEX positive droplets represent the total number of alleles. Ratio of FAM positive droplets to HEX positive droplets provides the fractional abundance of HDR edited alleles (Figure 7.5).

ddPCR HDR Genome Edit Detection Assays:

- Can be designed to detect single base pair substitutions, insertions, and deletions up to 25 bp
- Can be designed for donor homology arms ≤450 bp
- Are available with amplicon length-matched reference assays for human, mouse, and rat
- Quantify precise edits with sensitivity ≤0.5%
- Are quantifiable using QuantaSoft Analysis Pro Software

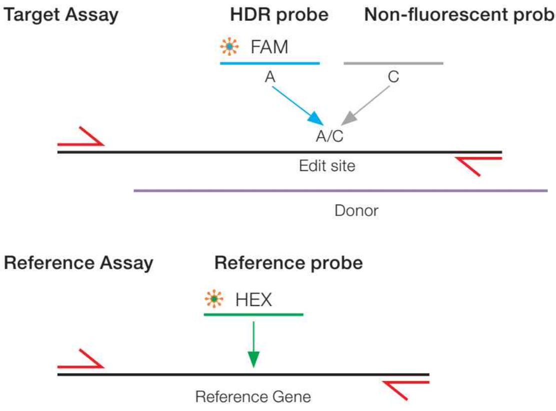

Target Assay
HDR probe
Non-fluorescent probe
FAM
A
C
A/C
Edit site
Donor
Reference Assay
Reference probe
HEX
Reference Gene

**Fig. 7.4. HDR assay design.**

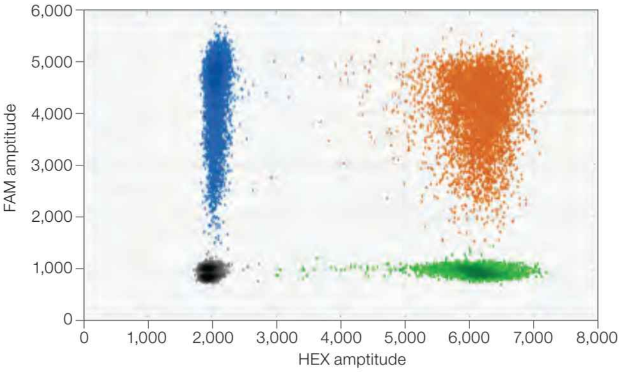


**Fig. 7.5. HDR assay readout.** HDR mutation positive control (WT + 20% gblocks gene fragment containing a single base pair substitution) with HDR GED + HDR REF Assay. The HDR edits are FAM+HEX-, while the HEX+ and FAM+HEX+ positive clusters represent the total number of copies, including WT, NHEJ, and HDR edits.

## Detection of NHEJ Edits Using ddPCR NHEJ Genome Edit Detection Assays

NHEJ assays are duplexed primer probe-based ddPCR assays (Figure 7.6). A FAM-labeled probe is designed to bind a reference sequence distant from the nuclease target site but still within the amplicon. A second HEX NHEJ/drop-off probe binds WT sequence at the nuclease target site, such that NHEJ-induced indels block the probe from binding. In a 2-dimensional view of the ddPCR analysis, droplets with signal from both the FAM and HEX probe contain wild-type amplicons while droplets that are FAM-positive but HEX-negative contain amplicons with mutations at the target site (Figure 7.7). The ratio of FAM positive to FAM/HEX double positive droplets provides the fractional abundance of NHEJ mutations.

ddPCR Genome Edit Assays for NHEJ:

- Detect indels up to 40 bp in length symmetrically on either side of the cut-site, or 80 bp asymmetrically
- Quantify precise edits with sensitivity ≤0.5%
- Are quantifiable using the “drop-off assay” analysis option in QuantaSoft Analysis Pro

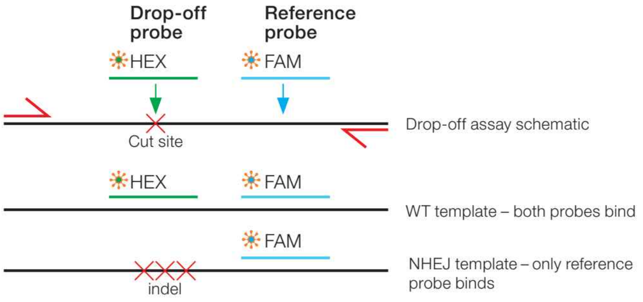


**Fig. 7.6. NHEJ assay design.**

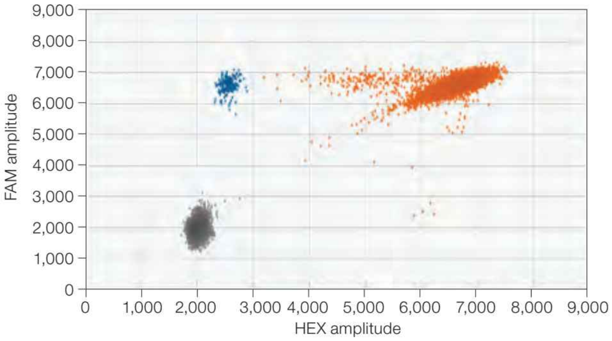


**Fig. 7.7. NHEJ assay readout.** NHEJ mutation-positive control (WT + 1% gblocks gene fragment containing a one base pair deletion at the predicted cut site). The WT cluster is positive for both FAM and HEX, while the NHEJ mutant single-positive droplets are positive for FAM only.

# 8 Next-Generation Sequencing Library Analysis

## Overview

Next-generation sequencing (NGS) systems are extremely sensitive to the quantity of library loaded in the sequencing run. Overloading frequently produces unusable data and underloading wastes reagents and time. Droplet Digital™ PCR (ddPCR™) complements NGS by offering accurate library concentration measurements and unique quality analyses that are not available with other methods.

Standard methods for quantifying NGS libraries have disadvantages. Electrophoresis and spectrophotometry measure total nucleic acid concentrations, whereas optimal cluster density or template-to-bead ratio depends on the appropriate concentration of PCR-amplifiable DNA molecules. Incorrectly adapted products or adapter-adapter dimers cannot be distinguished easily from bona fide library fragments. These undesirable species can compete for binding sites on the flow cell, inhibit cluster formation, increase the likelihood of suboptimal loading, and reduce the total number of high-quality reads. These methods also have low sensitivity, consuming nanograms of precious samples, and are not suitable for high-throughput workflows. A more accurate method to quantify libraries before sequencing is required to maximize usage of sequencing platforms.

ddPCR provides an absolute, standard-free method to measure library concentration and quality, while optimizing overall NGS performance. ddPCR makes very precise and accurate measurements of the library stock concentrations for flow-cell loading. The digital nature of droplet partitioning is conducive to accurate quantification by reducing competing PCR reactions.

# ddPCR Quantification on Illumina TruSeq v2 Chemistry

For the Illumina MiSeq and HiSeq Platforms, the total possible reads is directly related to the concentration of prepared library loaded. These platforms have a narrow loading concentration range requirement for successful runs. To maximize the sequencing information from a given sequencing run, accurate measurements of library concentration must be made.

Measuring concentration by ddPCR before amplification may help determine the number of additional PCR cycles needed, if any, to obtain enough library for loading.

Measuring concentration by ddPCR before the library is loaded for sequencing determines concentration and helps identify any library construction quality anomalies accumulated during the process.

The TruSeq v2 library preparation protocol is shown in Figure 8.1. TruSeq Y-adapters, containing both P5 and P7 sequences, are ligated to library DNA inserts. Following PCR amplification, the resulting amplicons contain P5 and P7 sequences directionally oriented on either strand of the fragment library.

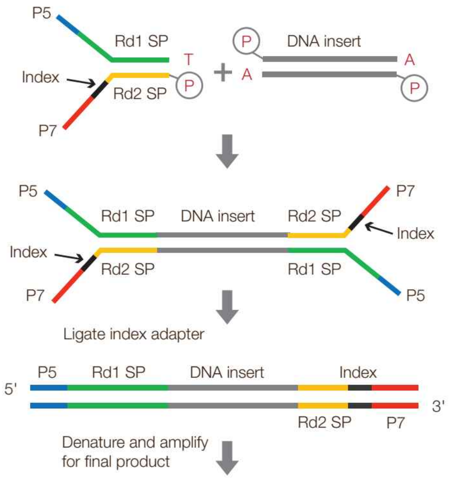

P5
Rd1 SP
T
P
DNA insert
A
Index
Rd2 SP
P
+
A
P
P7
P5
Rd1 SP
DNA insert
Rd2 SP
P7
Index
Index
Rd2 SP
Rd1 SP
P7
P5
Ligate index adapter
P5
Rd1 SP
DNA insert
Index
5'
3'
Rd2 SP
P7
Denature and amplify
for final product

**Fig. 8.1. TruSeq v2 library preparation.**

Our duplex assays target the flanks of the library fragments with two probes targeting both the P5 and P7 moieties. We can directly measure linkage between probes 1 and 2 because they are co-localized in the same droplet (Figure 8.2). Targeting both flanking segments ensures testing for both well-formed and poorly formed fragments, allowing quantification of species possessing both adapter arms.

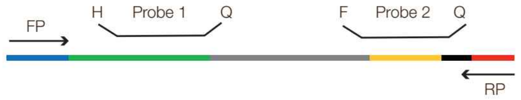


Fig. 8.2. Design of the ddPCR Library Quantification Kit for Illumina TruSeq assay.

An example of the 2-D plot of ddPCR FAM versus HEX data observed for this assay is shown in Figure 8.3.

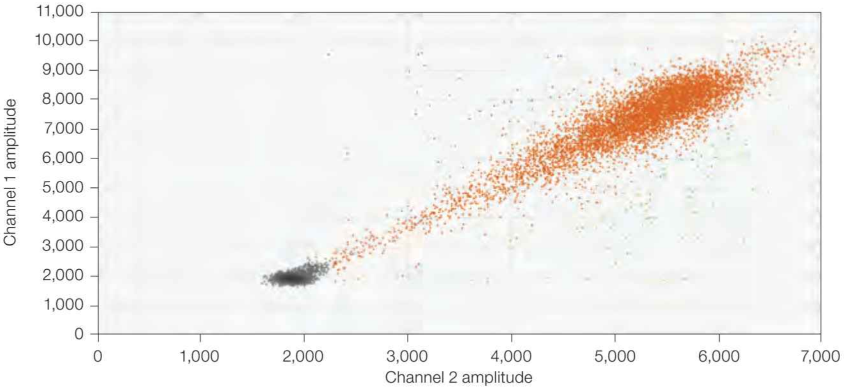


Fig. 8.3. 2-D plot of ddPCR data from the ddPCR Library Quantification Kit for Illumina TruSeq.

# Library Quality Analysis

The ddPCR Library Quantification Kit for Illumina TruSeq contains TaqMan assays designed to detect and quantify both the P5 and P7 adapter arms. Each assay can be used to independently confirm the formation of bona fide library fragments by exploiting the ability of the QX100™ or QX200™ Droplet Digital PCR System to detect subpopulations of templates with differing amplification efficiencies and with different combinations of P5 and P7 moieties.

A 2-D plot of the library can be used to provide information on the quality of the library (Figure 8.4). Inserts of varying sizes within the library can be observed along a diagonal path, with larger fragments near the negative droplets and smaller inserts in the upper-right corner. This is because the smaller amplicons produce greater fluorescence during PCR amplification. This characteristic pattern enables you to assess the quality of your library. At the highest point in the upper-right corner is a population that appears separate from the bulk of the library. This population is the adapter-dimer population with no inserts (red circle in Figure 8.4).

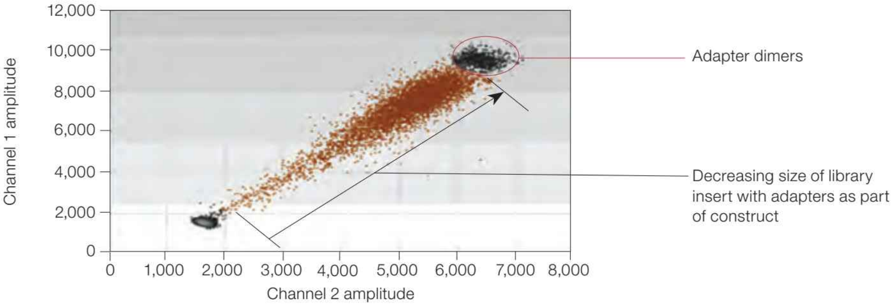


Fig. 8.4. Additional library information produced by ddPCR.

Droplets that appear above and below the insert population (large, diagonal cluster of increasing fluorescence) represent rare species with three or more adapters ligated to the insert (Figure 8.5). These populations can be selected in QuantaSoft™ Software using the lasso function. By selecting the desirable bulk population that lies along the diagonal (green circle, Figure 8.5), excluding the adapter-adapter population in one color channel (blue circle, Figure 8.5), and excluding the undesirable side populations (adapter-adapter and extra adapter populations in red circles, Figure 8.5), you can then use the Ratio tab in QuantaSoft Software to select **Fractional Abundance (a/a+b)**, and get a readout of the percentage of your library with inserts. This can be a quick and easy readout of the quality of your libraries. As anything with two adapters can still contribute to your cluster densities, you must include the adapter-adapter populations in your quantification and balancing of your libraries for sequencing. This quality measurement can be used to enable you to appropriately load and balance your sequencing runs, thus compensating for poorly formed library fragments and improving greater reading depth.

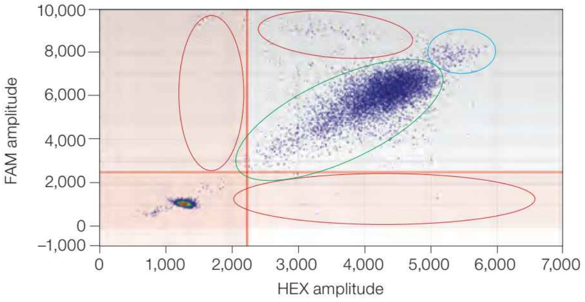


**Fig. 8.5. Various species visualized by the ddPCR Library Quantification Kit assay.**

The library quality information obtained from 2-D plots will fuel further investigations into improvements of NGS sequencing, possibly by determining library fragment PCR efficiencies. Improvements in the NGS workflows will likely result from extremely accurate quantification by ddPCR. For example, you may be able to eliminate secondary amplification steps if enough library material is generated for sequencing directly after library construction, thus avoiding unnecessary steps and further skewing of fragment representation.

# Next-Generation Sequencing Reads

In Figure 8.6, we demonstrate the relationship between the ddPCR-determined library concentration loaded and the number of total reads from sequencing on a MiSeq Platform. By measuring library concentrations, the amount of input can be balanced across samples before loading the NGS instrument.

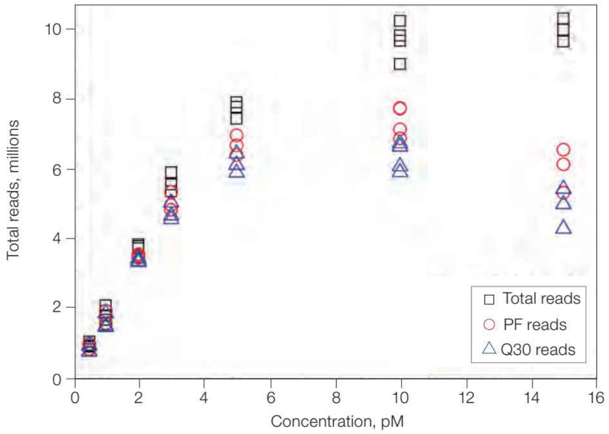


**Fig. 8.6. Plot of the reads vs. input library concentration.** Impact of input library concentration on total usable reads. Cluster density at 5 pM was approximately 800,000/mm$^2$. PF, passing filter.

As the cluster density and therefore number of reads is intimately tied to the loading concentration of the prepared library, moderate differences can compromise read capacity and quality of the MiSeq Platform. NGS library quantification with ddPCR is extremely accurate, providing accuracy better than 15% with a confidence level of 95%. This method provides absolute quantification, eliminating the need to develop standards. Use of ddPCR in NGS significantly increases reliability and quality, and optimizes use of consumables, labor, and instrument time. Additional information, not available with other methods, such as adapter-adapter dimers and improperly adapted species can be seen. We have observed an inverse relationship between the size of the amplicons and fluorescence intensity with our ddPCR Library Quantification Kit for Illumina TruSeq. The smaller the amplicon size, the higher the fluorescence attained, most likely due to PCR efficiency within the droplets. This information-rich content provides you with a digital quality check in the library construction before a sequencing run.

## Library Balancing

When performing NGS on the MiSeq Platform, it is important to aim for a cluster density of approximately 800,000/mm$^2$ for optimal performance. ddPCR measurements can be used to establish the functional relationship between input library concentration and the number of usable reads on the MiSeq Platform.

We examined the precision of ddPCR in balancing 12 TruSeq DNA libraries from human genomic DNA using concentration measurements obtained from the QX100 System using the ddPCR Library Quantification Kit (Figure 8.7). Based on the ddPCR concentration measurements, libraries with an average fragment length of 447 bp could be balanced within less than 15% of each other with a confidence interval of 95%. Similar balancing results were observed when RNA-Seq libraries with an average fragment length of 280 bp were used.

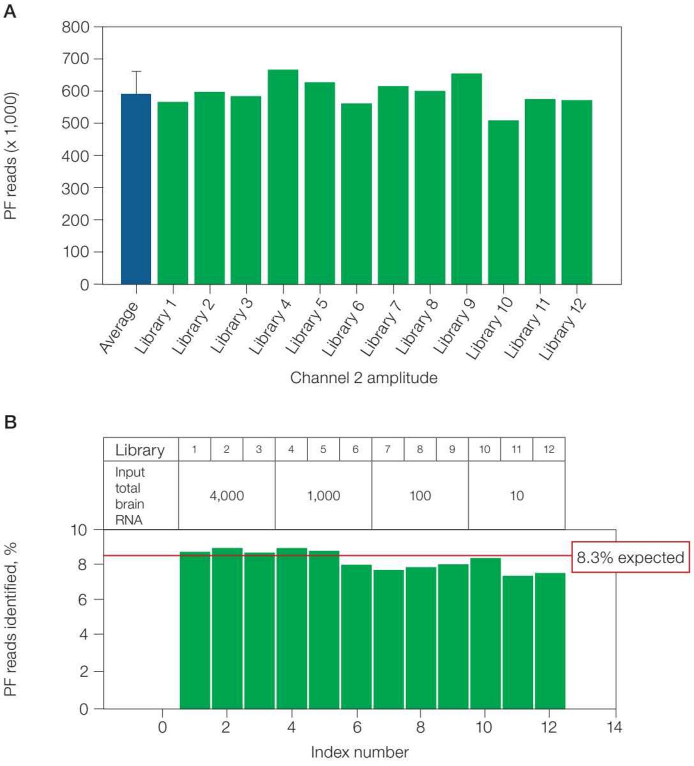


**Fig. 8.7. ddPCR library balancing results.** PF, passing filter.

## Amplicon Recovery from Droplets

Bio-Rad's ddPCR Supermix for Probes (no dUTP) has been optimized for PCR amplification of rare target DNA sequences and for NGS library preparations. The droplet PCR supermix provides unbiased amplification and greater template coverage of sequencing template.

Generate droplets by following the ddPCR standard workflow and protocols. If your goal is to read droplets as well as recover material from droplets, make the desired number of wells to be read on the QX100 or QX200 System (nonrecoverable), and also make replicates to be broken open (not to be read on the QX100 or QX200 System).

For example, a column of wells could be generated (eight wells), four of which are read after PCR and four of which are not read. In QuantaSoft Software, set up the plate where only four of the eight wells are read. After the QX100 or QX200 System has finished the run, remove the plate and pierce the foil of the four remaining unread wells and proceed with breaking the droplets from those wells.

If your goal is to generate droplets and break them open only after PCR, without reading them on the QX100 or QX200 System, then proceed directly with the following protocol for recovery of DNA from droplets after PCR:

1. Pipet out the entire volume of droplets and oil from a well into a 1.5 ml tube (combine up to ten replicates if desired).
2. Pipet and discard the bottom oil phase after droplets float to the top.

3. Add 20 µl of TE buffer for each well used; add additional TE buffer by multiplying by the number of combined replicate wells if applicable.
4. In a fume hood, add 70 µl of chloroform for each well and cap the tube. Add additional chloroform by multiplying by the number of combined replicate wells if applicable.
5. Vortex at maximum speed for 1 min.
6. In a centrifuge, spin down at 15,500 x g for 10 min.
7. Remove the upper aqueous phase by pipetting, avoiding the chloroform phase, and transfer it to a clean 1.5 ml tube (this is the recovered DNA).
8. Dispose of the chloroform phase appropriately.
9. If desired, estimate size using Bio-Rad's Experion™ DNA 1K Analysis Kit Chip and/or requantify by ddPCR.

In Figure 8.8, the contents in the Eppendorf Tubes following the droplet breaking protocol demonstrate the layers formed by chloroform and the broken aqueous layer. The upper aqueous phase can readily be pipetted into a clean tube for downstream analysis.

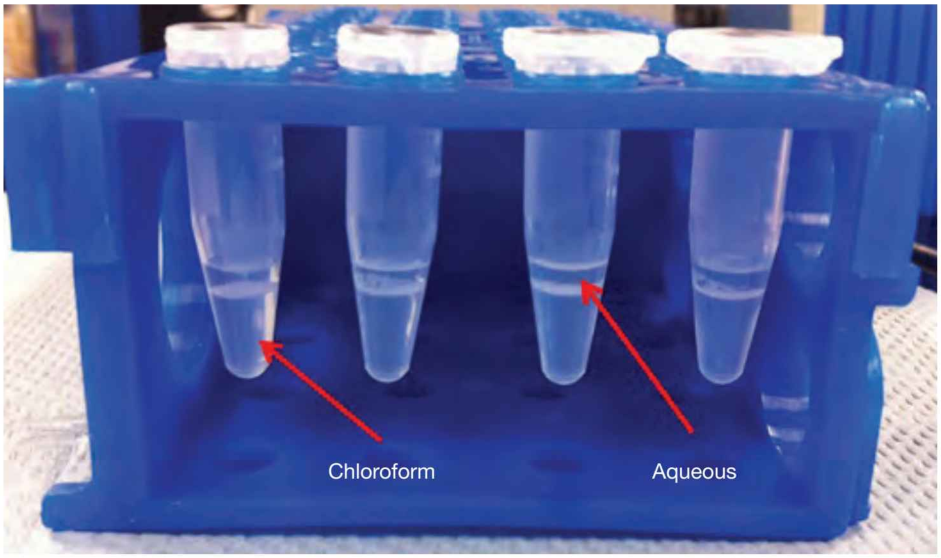
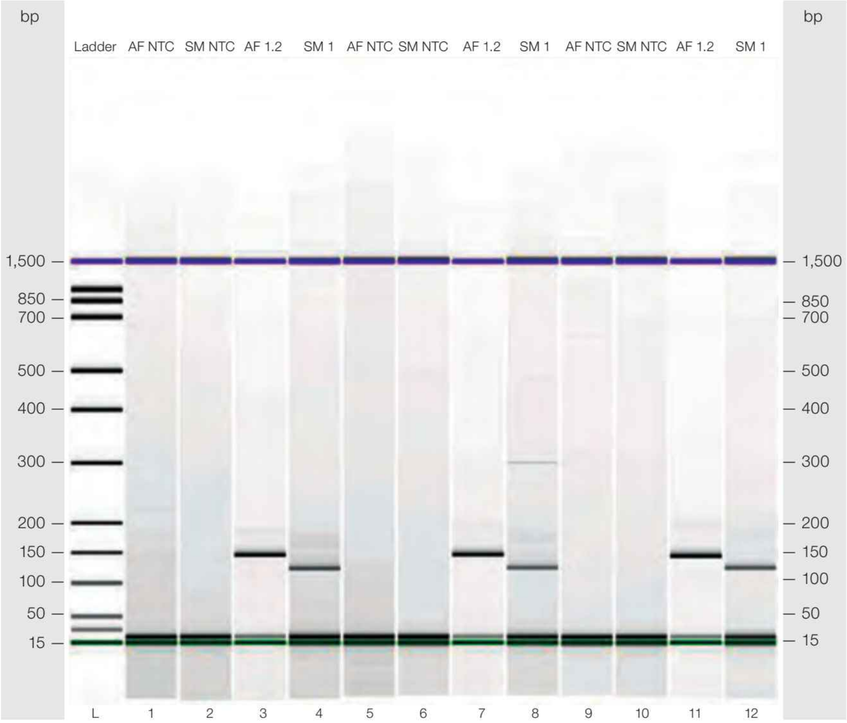


**Fig. 8.8. Aqueous phase recovery following droplet amplicon recovery protocol.**

Recovered DNA can be analyzed by gel electrophoresis (Figure 8.9), sequencing, and ddPCR (Figure 8.10).


bp
Ladder
AF NTC
SM NTC
AF 1.2
SM 1
AF NTC
SM NTC
AF 1.2
SM 1
AF NTC
SM NTC
AF 1.2
SM 1
1,500
850
700
500
400
300
200
150
100
50
15
L
1
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4
5
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8
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11
12

**Fig. 8.9. Postrecovery analysis of ddPCR products.** bp, base pair; NTC, no template control.

In Figure 8.9 four replicate wells are shown for each assay, of which three were combined for the droplet breaking protocol, and the fourth was read by the QX100 Droplet Reader. Gel analysis and electropherograms of amplicon products made from two different reactions prepared in droplets and no template controls are shown in the panels to the left. Lanes 1, 2, 5, 6, 9, and 10 are no template controls of the reactions; lanes 3, 7, and 11 are a *Staphylococcus aureus* assay; and lanes 4, 8, and 12 are a different assay for the *S. aureus* template.

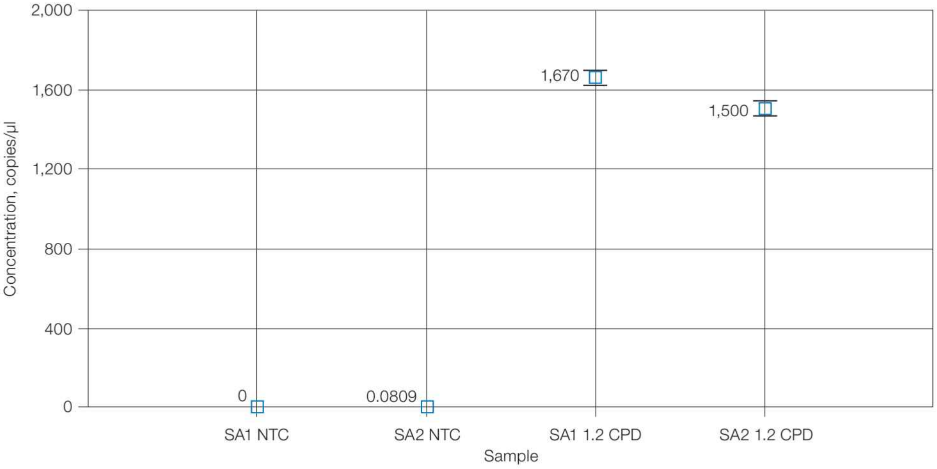


**Fig. 8.10. ddPCR quantification of droplet recovered amplicons.** CPD, copies per droplet; NTC, no template control; SA1, *S. aureus* assay 1; SA2, *S. aureus* assay 2.

Results of ddPCR concentration plots for the two *S. aureus* reactions carried out in the example are shown.

# 9 Additional Applications

## Linkage Analysis

Physical linkage of two alleles in Droplet Digital™ PCR (ddPCR™) can be thought of in at least two ways:

- Tandem repeats of the same sequence
- Proximity of two sequences (targets) physically linked on the same piece of DNA

We recommend using restriction enzymes to digest the DNA in a copy number variation (CNV) study and physically separate the two target copies (in order to ensure random target distribution into droplets). If the copies are not separated, then the target can be undercounted because the copies will always segregate into the same droplet. This is especially true for tandem copies near each other. The extreme sensitivity of ddPCR, however, allows you to study linkage between targets and to infer the haplotype of samples by comparing digested vs. undigested DNA.

Figure 9.1 represents a study of different human samples using restriction digestion in comparison with undigested DNA for the same sample for the *MRGPRX1* gene, which has variable copy numbers (CNs) in different populations. In samples 4 and 5, the undigested (uncut) samples have lower CNs compared to the digested (cut) DNA of the same sample, which suggests the copies in those samples are linked on the same chromosomal strand.

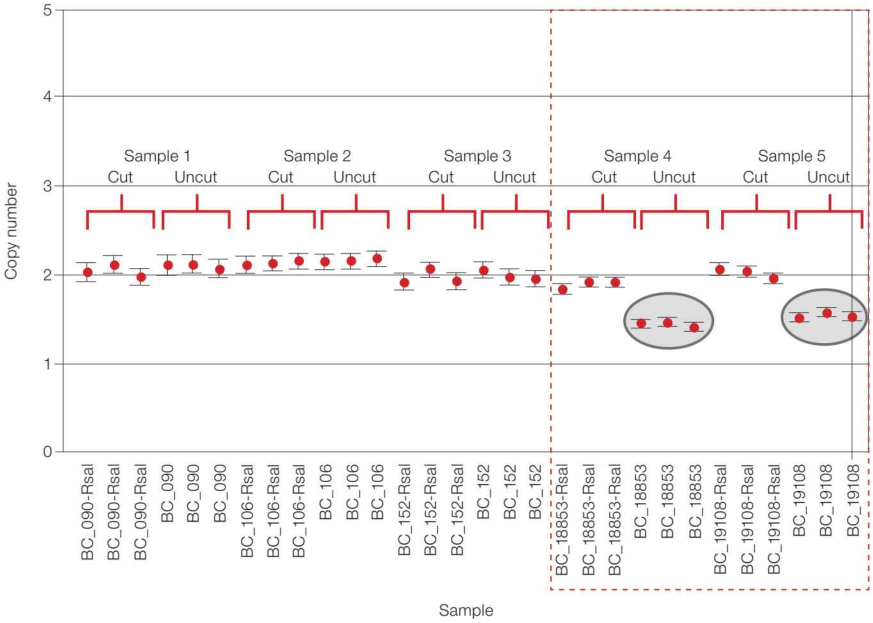


**Fig. 9.1. CNV of different human samples using restriction digestion vs. undigested DNA for the same sample for the *MRGPRX1* gene.**

## Milepost Assay

Linkage studies can also provide information on the quality of the template, and allow you to determine how degraded or fragmented the template might be by examining an assay that serves as a positional anchor and a second assay located at a determined distance on the same chromosome. This is known as a milepost assay.

Figure 9.2 represents the assay design scheme for performing a milepost assay. Different DNA targets are designed at different lengths from an anchor assay, typically 1 kb, 10 kb, and 100 kb. Each milepost marker is run in multiplex with the anchor assay.

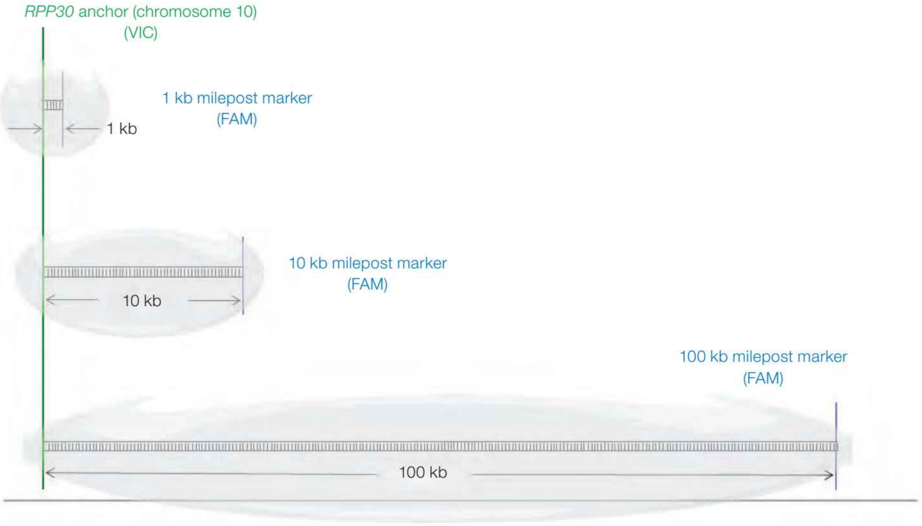

*RPP30* anchor (chromosome 10)
(VIC)
1 kb milepost marker
(FAM)
1 kb
10 kb milepost marker
(FAM)
10 kb
100 kb milepost marker
(FAM)
100 kb

**Fig. 9.2. Assay design for the milepost assay.**

Demonstration of a milepost assay is presented in Figure 9.3, which shows the 2-D plots of FAM amplitude and VIC amplitude for an RNaseP anchor assay (VIC), and progressively farther assays on chromosome 10 for a human DNA sample. The upper left panel demonstrates a control to account for the inherent probability of two copies completely separated from each other (RNaseP, which is on chromosome 10, and an assay located on chromosome 6) randomly co-localizing in the same droplet. The remaining panels demonstrate that as the distance between the anchor and the milepost assay (on the same chromosome as RNaseP) increases, the number of double-positive droplets decreases. At a distance of 100 kb, the double-positive population is equal to the unlinked control. Finer resolution could be done with more milepost assays to cover the range.

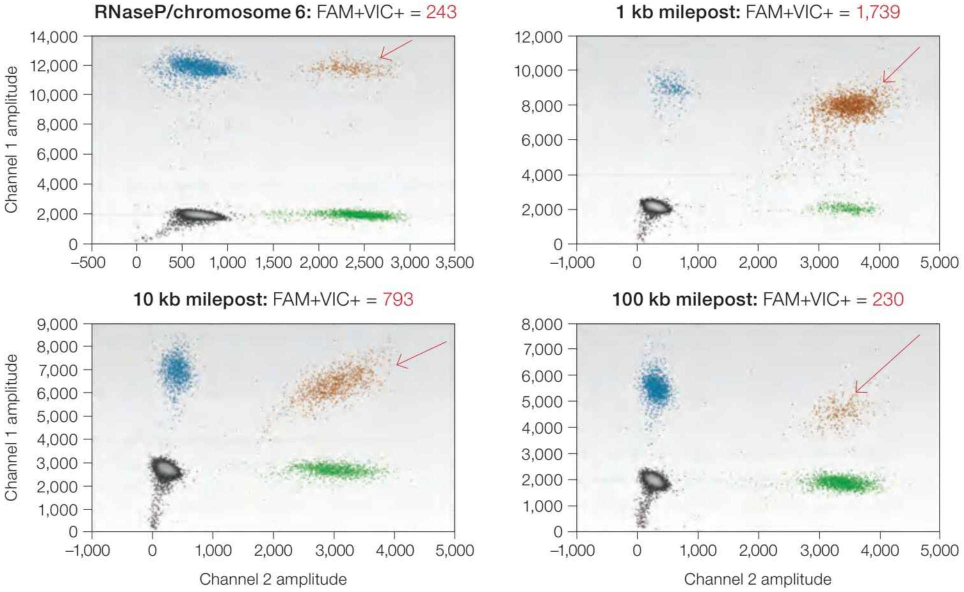


Fig. 9.3. Milepost assay results to determine the quality of DNA by a linkage study.

## microRNA Amplification by ddPCR

A method for absolute quantification of microRNA (miRNA) described here shows the day-to-day reproducibility of a tenfold change in the starting quantity of synthetic template mir-210. An example ddPCR amplification of an miRNA synthetic template (mir-210) was done in a two-step reaction. The reverse transcription (RT) reaction was done in bulk solution, and the cDNA was partitioned into droplets before PCR amplification.

miRNA synthetic template for mir-210 (RNase free, HPLC purified) was obtained from Integrated DNA Technologies, Inc. The miRNA synthetic templates in TE buffer at a final concentration of 1 µM were aliquoted into individual-use tubes and frozen at –80°C. Each miRNA stock was loaded in RNase-free water on ice just before performing an RT assay. Reverse transcription of the synthetic miRNA templates was done in bulk solution using the TaqMan microRNA Reverse Transcription Kit (Thermo Fisher Scientific), and using the miRNA RT protocol with the specific RT primers for each miRNA.

ddPCR amplification of the cDNA generated in the RT reaction was done by adding 1.33 µl of each cDNA to a 20 µl ddPCR reaction mixture containing 1 µl of 20x TaqMan miRNA Reagent (Thermo Fisher Scientific) specific to the miRNA of interest, 10 µl of Bio-Rad's 2x ddPCR supermix for probes, and 7.67 µl of molecular biology–grade water. The droplets were generated, thermal cycled, and detected using standard procedures.

## Day-to-Day Reproducibility Study: mir-210 miRNA

mir-210 synthetic templates at 1.6 nM originated from a single 1 µM stock. The 1.6 nM stock was used to create nine concentrations (10 µl into 90 µl RNase-free water) on ice. Concentrations 5–9 were used for the titration series, with an estimated concentration range of 2,135–0.21 copies/µl of ddPCR. The data are plotted as individual well replicates (duplicates) for each day with Poisson 95% confidence intervals in Figure 9.4.

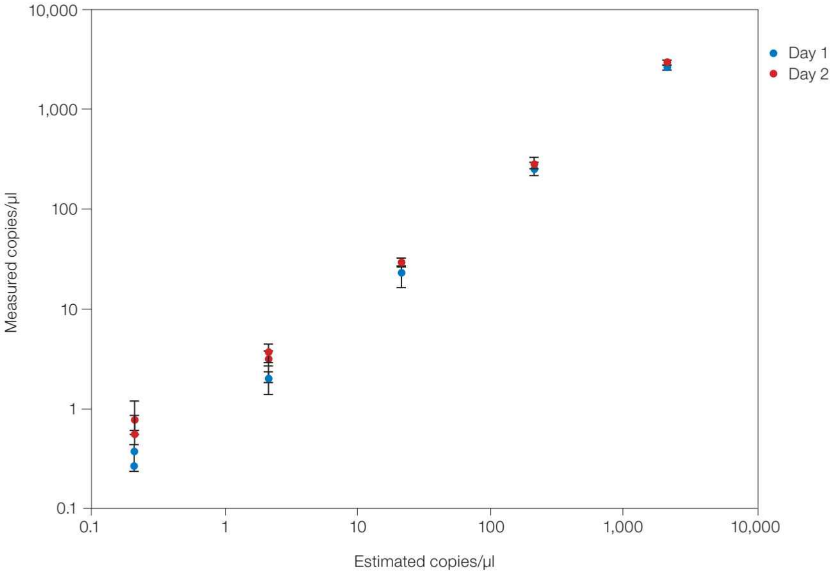


**Fig. 9.4. Day-to-day reproducibility of mir-210 miRNA assay: measured vs. theoretical.**

# Multiplexing

Various schemes can be designed to add complexity to assays run in ddPCR. For instance, you may want to add more than two assays to a reaction. The QX100™ and QX200™ Droplet Digital PCR Systems can detect fluorescence in two color channels (FAM and HEX).

However, there is an added dimension of fluorescence amplitude that can be used to multiplex more than two assays at a time.

The basic concept for multiplexing is to make use of the differences in fluorescence amplitude signal to change the spatial positioning of the droplet clusters in the 2-D data plots drawn in QuantaSoft™ Software. By varying the concentration of the TaqMan assay, the end-point fluorescence amplitude can be shifted in many assays. Due to this shift, you can include two or more assays in the FAM and/or HEX channels and empirically determine what concentrations will resolve the two assays in separate clusters.

A good starting point would be to halve, or conversely, double the concentration of an assay relative to the standard final 1x concentration in order to spatially resolve the two assays in a 2-D plot of FAM and HEX amplitudes.

Figure 9.5 is an assay targeting chromosome 10 of the human genome. The stock has the highest fluorescence amplitude and subsequent twofold changes in starting concentration demonstrate resolvable droplet populations based on fluorescence amplitude down to one-quarter dilution.

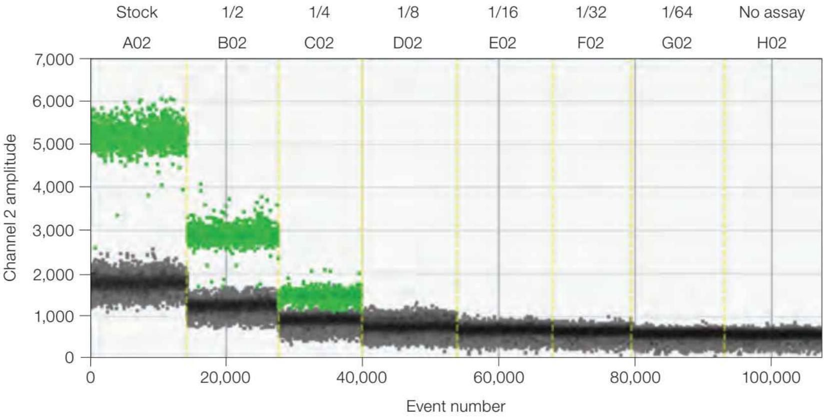


**Fig. 9.5. Dilution series of an assay targeting chromosome 10 of the human genome.**

Figure 9.6 represents an example of such a multiplexing strategy being used with one FAM channel assay and two HEX channel assays in a 2-D plot of the data.

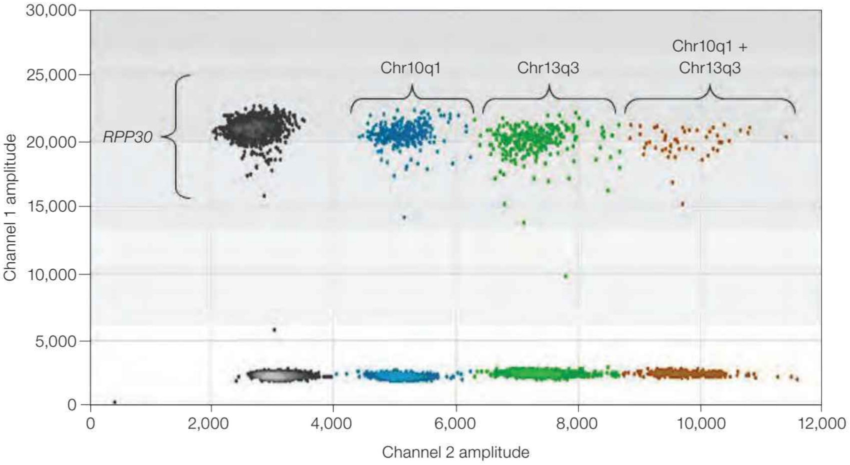


**Fig. 9.6. 2-D plot of FAM amplitudes (channel 1) vs. HEX amplitudes (channel 2) for a multiplex experiment.**

In this experiment, a single FAM assay was used (*RPP30*) at standard 1x final concentration and two different HEX assays at different concentrations (Chr13q3 and Chr10q1). Assay Chr10q1 was used at 1/2 x standard concentration and assay Chr13q3 was used at 1x final concentration in the reaction. All three assays were present along with template in the supermix before making droplets. Using tools in QuantaSoft software to manually group populations, the four upper droplet clusters (gray, blue, green, orange) represent those droplets pertaining to droplets that are positive for the *RPP30* assay. The blue clusters are those droplets that are positive for the Chr10q1 assay. The green clusters are those droplets that are positive for the Ch13q3 assay. The orange clusters are those droplets that are positive for both Chr10q1 and Chr13q3 simultaneously. Negative droplets for the *RPP30* assay are the lower four droplet clusters (gray, blue, green, orange).

# 10 Droplet Digital™ PCR Tips, Assay Considerations, and Troubleshooting

## Assay-Dependent Cluster Shifts

As with any PCR-based technology, assay design and sample preparation are important for obtaining good quality data. Before running a Droplet Digital PCR (ddPCR™) experiment, know the goal or possible expected outcomes of the experiment because different types of experiments require different controls, sample preparation, amounts of DNA or RNA, and data analysis.

### Shifted Clusters Due to Probe Cross-Reactivity

If you see a shift inwards or upwards on the 2-D plot, this is most likely probe cross-reactivity. Probe cross-reactivity occurs when a probe binds to a nonperfect sequence and undergoes cleavage (Figure 10.1). This is more common in rare mutation detection (RMD) assays (for example, single nucleotide polymorphism [SNP] assays), where the two probes differ by only one base. The amount of nonspecific probe cleavage is a function of how close the melting temperature ($T_m$) of the mismatched probe is to the annealing/extension temperature used for the assay. For this reason, RMD assays are designed to maximize the $T_m$ difference between a perfectly matched and a mismatched target. A cross-reacting probe causes single positive clusters (Ch1+/Ch2– and/or Ch1–/Ch2+) to migrate toward the axis of the other channel, which is most easily viewed in the 2-D amplitude plots (see Figure 10.1A). Figure 10.1 exemplifies this phenomenon where the FAM probe cross-reacts to a PCR product perfectly complemented by the VIC probe, resulting in mild to moderate FAM probe cleavage in the droplets. This causes an upward shift (or migration) in the location of the droplets in 2-D space. A similar shift is observed for the FAM+ droplets. These shifts are clearly seen in the 2-D amplitude plots. By contrast, the 1-D amplitude plots and histograms of these same data are difficult to interpret (Figures 10.1B–E). To avoid such confusion, always classify the droplets of cross-reacting assays while viewing the 2-D amplitude plots. For users operating QuantaSoft™ Software Version 1.2.10 or earlier, appropriate droplet classification using a linear threshold may not be possible without misclassifying some droplets. Upgrading to QuantaSoft Software Version 1.3.2 or higher allows for proper classification using the clustering tools.

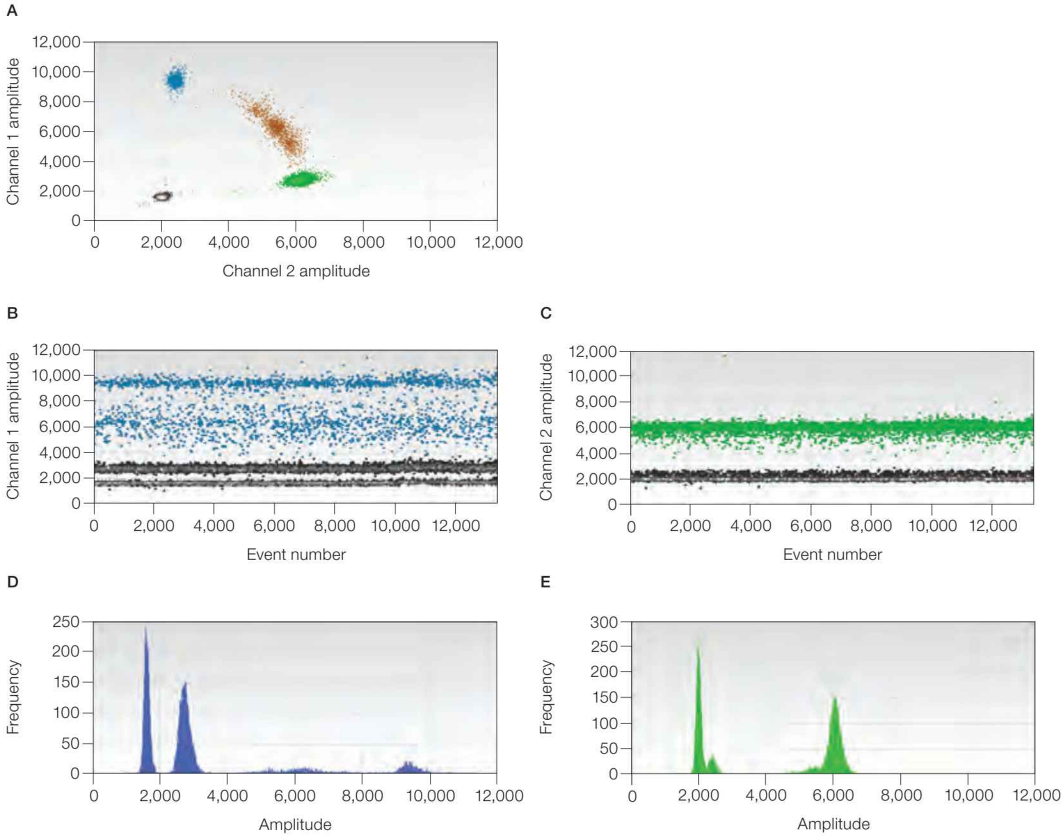


**Fig. 10.1. Cross-reacting probes in a rare mutation detection assay.** 2-D amplitude (**A**), 1-D amplitude (**B** and **C**), and histogram (**D** and **E**) plots.

## Probe Cross-Reactivity Can Identify Off-Target Amplification

An unexpected extra cluster of positive droplets with fluorescence intensity less than the cluster containing the target of interest can be caused by a sequence variant in the target of interest (Figure 10.2). The droplets that cluster around 10,000 relative fluorescence units (RFU) contain a variant DNA sequence that is not perfectly matched to the designed probe. The perfectly matched sequence is the higher cluster around 12,000 RFU.

Often the distinction among two or more positive clusters is desirable because it provides additional information regarding the sample. If the mid-level cluster represents the detection of a potentially functional homolog, consider setting the threshold below this cluster to include it in the quantification, or lowering the annealing temperature so that these two clusters merge into one cluster. If the mid-level cluster is not desired, consider setting the threshold above this cluster to exclude it from target quantification.

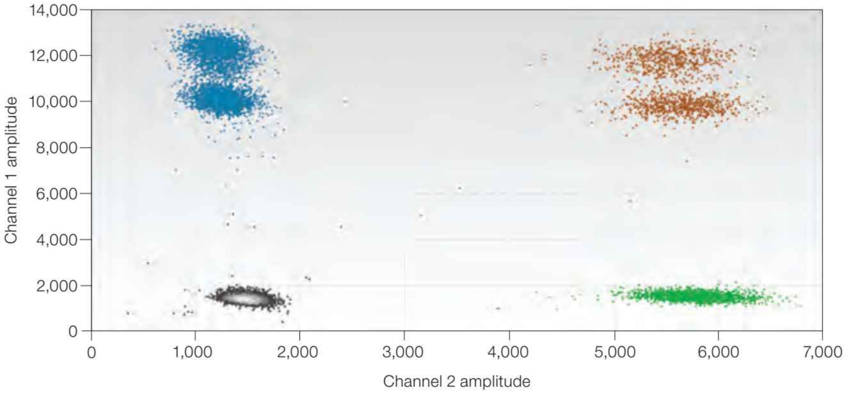


**Fig. 10.2. Extra droplet clusters.**

To prevent the off-target amplification, try increasing the annealing temperature of the PCR reaction to improve specificity (Figure 10.3), or alternatively, try digesting the sample with a restriction enzyme that will cut the nonspecific target while preserving the specific target. This latter approach requires some knowledge of the nonspecific target's sequence. Assay redesign can often be used to remove subclusters. Consider using Primer-BLAST to determine whether one of the primers is potentially nonspecific. If this is the case, examine whether a primer redesign would avoid the amplification of this product. If no flexibility exists in primer design, shift the probe to minimize complementarity to the off-target sequence.

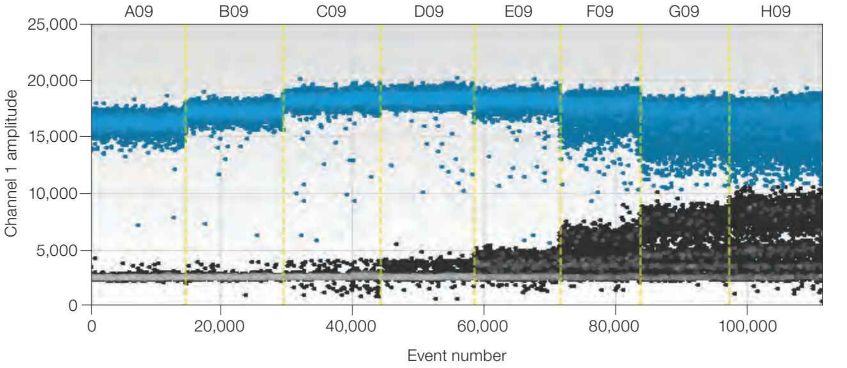


**Fig. 10.3. Annealing/extension gradient where the assay detects an off-target species.**

## Positive Droplets in No Template Control Wells

Digital PCR can detect very low levels of target DNA so it is important to prevent template/amplicon contamination and to run no template controls (NTCs). Positive droplets in NTC wells that are at intensities equal to those of positive droplets in sample wells are typically caused by template or PCR product (amplicon) contamination in the reagents. Having a clean environment and clean NTC wells (that is, no positive droplets) is imperative when the application is rare sequence detection (wells with a low number of positives). In Figure 10.4, the first well (a contaminated NTC well) has four droplets of the same amplitude as those seen in the well on the right (positive sample).

If positive droplets in NTC wells occur, make sure that good laboratory practices for PCR are being followed in the laboratory (Kwok and Higuchi 1989).

Suggested guidelines are as follows:

- Wipe down pipets, tip boxes, and benchtops with 5–10% bleach
- Prepare master mixes in a template-free environment, add samples and generate droplets in an amplicon-free environment, perform PCR, and read droplets in a room separate from the sample preparations
- Do not reuse DG8™ Droplet Generator Cartridges, oils, gaskets, plates, or pipet tips
- Wear appropriate personal protective equipment that is discarded or confined to appropriate locations (that is, template-free room for master mix assembly, amplicon-free room for template addition and droplet generation, and PCR and post-PCR rooms for droplet reading)

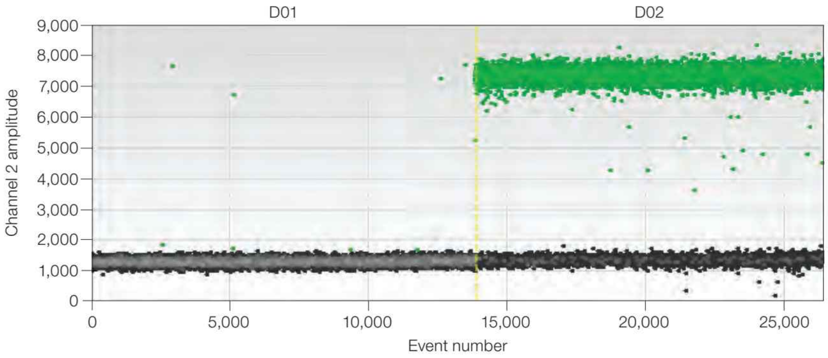


**Fig. 10.4. An example of contamination of an NTC well (left) with amplitudes similar to a positive template reaction (right).**

If desired, dUTP-containing supermixes (ddPCR Supermix for Probes) can be used in conjunction with heat-labile uracil N-glycosylase (UNG) or uracil DNA glycosylase (UDG) to reduce the potential for false positives resulting from the presence of previously amplified products. Add the UNG at 0.05 units/20 µl of ddPCR reaction mixture and create droplets as normal. Transfer the foil-sealed 96-well PCR plate containing droplets to the thermal cycler and add a 30 min 37°C incubation step in front of the standard recommended thermal cycling protocol. During this incubation period, UNG will digest U-containing products, such as those from past experiments performed with the ddPCR supermix for probes. UNG is heat inactivated during the first 3–10 min of the 95°C initial PCR hot-start step in the standard PCR protocol.

**Note:** The UNG approach can address contamination caused by PCR products created using ddPCR Supermix for Probes or One-Step RT-ddPCR Kit for Probes; however, it will not address contamination caused by sample-source templates or PCR products created using droplet PCR supermix. Ultramers or long oligo PCR templates are especially problematic because the stock concentration is typically very high and can easily spread to pipets and other surfaces. It can be useful to order ultramers with uracils (Us) in place of a few of the thymidines (Ts) in order to take advantage of UNG treatment in case contamination becomes a problem.

## High Mean Fluorescence Amplitude Intensity

If the fluorescence amplitude of negative droplets is excessively high such that they are all considered positive and therefore concentration cannot be determined, it is possible the sample's target concentration is so high that every droplet contains DNA target and no negative droplets exist (Figure 10.5). When there are no negative droplets, Poisson correction cannot be applied and it is not possible to calculate a concentration.

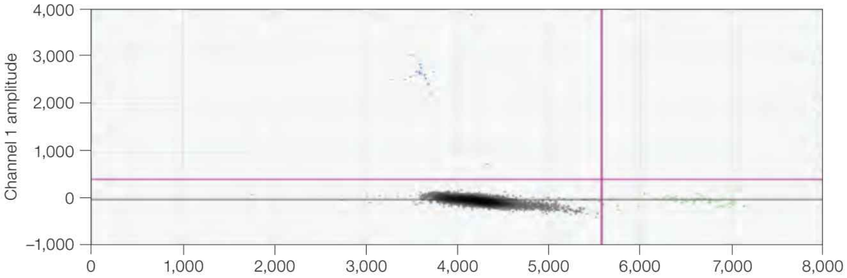


**Fig. 10.5. Example of all droplets being positive (containing template) for the FAM assay and/or the VIC assay.**

Having all positive droplets could arise from multiple issues.

1. Polymerase independent — probe hydrolysis due to poor long-term storage of probe stock solution, such as in a nonbuffered solution (for example, water) at 4°C.
    - Reorder the probe and make probe stock solution with 10 mM Tris, pH 8.0–8.5, and store at –20°C
2. Polymerase dependent — assay components interact with each other in a way that results in premature probe cleavage by the enzyme.
    - Identify intra-assay interactions and redesign causative component(s) to reduce binding and cleavage

**Note:** Run an NTC well to identify this problem. If the negative droplets in the NTC well do not have high fluorescence amplitude droplets, then the target concentration is too high. If the negative droplets in the NTC well contain high amplitude droplets, the cause is either polymerase-independent probe hydrolysis or polymerase-dependent intra-assay interactions.

## No or Few Positive Droplets

If a new, never-before-tested assay fails to give positive droplets, consider the following:

1. The selected restriction enzyme may have cut within the target locus.
    - Recommendation: test the assay against DNA digested with a different restriction enzyme as well as undigested DNA
2. The target locus resides in a region that contains secondary structure.
    - Recommendation: use restriction enzymes to cut the sequences surrounding the region to be amplified in order to limit the number of possible interactions with nearby nucleotides
3. The assay does not work at the predicted temperature.
    - Recommendation: first perform an annealing/extension temperature gradient to determine the temperature at which the assay works
4. The ddPCR reaction mix was not assembled correctly or the probe/primers were not ordered correctly.
5. One of the assay components was designed incorrectly or a mistake was made during synthesis.

## No or Low Total Droplet Count

To determine your droplet count, select the well in setup, click **Analyze**, then click the **Events** tab and make sure **Total** is selected. If the total accepted events or droplet counts are less than 10,000 consider the following recommendations:

- Use the recommended concentration of primer (900 nM), probe (250 nM), and 1x master mix. The QX100™ and QX200™ Droplet Digital PCR Systems are compatible only with Bio-Rad's ddPCR supermixes. Using less than the recommended concentration of any of these components may lower your droplet count
- Load the DG8 cartridge with the appropriate volumes of sample and droplet generation oil (20 µl and 70 µl, respectively). If less than 20 µl of sample is loaded, fewer droplets will be generated. Be sure to load the sample before the oil
- Use only purified nucleic acids. Any particulate matter (for example, residual fibers from sample preparation columns or beads) in the sample should be removed before assembling the ddPCR reaction mixture because these particulates can clog the DG8 Cartridge's microfluidic channels and disrupt droplet generation. To remove particulates from purified nucleic acids, spin the sample at 10,000 x g for 1 min and transfer the supernatant to a clean tube

- Do not exceed the recommended DNA load (66 ng/well undigested DNA or 1,500 ng/well digested DNA)
- Use only approved plates (ddPCR 96-Well Plates, Bio-Rad catalog #12001925 and Eppendorf twin.tec Semi-skirted 96-well Plates, catalog #951020362) with approved pierceable foil heat seals (Bio-Rad catalog #1814040)
- Properly seal the 96-well plate. Under- or over-sealed plates result in oil evaporation during thermal cycling and compromise droplet data quality. If using the PX1™ PCR Plate Sealer (Bio-Rad catalog #1814000), seal plates at 180°C for 5 sec. Do not use the PX1 sealing protocol twice on the same plate because this often disrupts the original seal
- Ensure that the full volume of the generated droplets is transferred into the 96-well plate by inspecting the DG8 Cartridge after transfer
- Use only approved pipet tips for droplet generation and droplet transfer. Rainin and Eppendorf tips are approved for use

For sample loading, use P-20 pipet tips and slowly dispense the sample into the bottom of the DG8 well rather than pipetting at the top edge of the well. Then dispense 70 µl of oil into the oil wells. Begin droplet generation within 2 min of oil loading.

Use a manual P-50 pipet with a normal bore P-200 tip (not wide or narrow bore) to transfer droplets. Angle the P-200 tip in the well to prevent the droplets from having to squeeze between the pipet tip and well bottom (angle the tip position such that it is not vertical in the well). Slowly draw 40 µl of droplets into the pipet tip over ~5 sec. Typically ~5 µl of air will be pulled into the tip, which helps prevent the oil from leaking out.

Position the pipet tip (containing the droplets) near the bottom of the well and dispense the sample, ensuring ample room between the well and the pipet tip so that the droplets do not shear upon dispensing.

## Inconsistent Concentration Results

Technical replicates of the same sample should yield concentration estimates that are within the Poisson confidence error bars 95% of the time. If the concentration estimates between technical replicates are not close, the most common causes are poorly mixed reaction mixtures or poor thermal cycler temperature uniformity.

### Insufficient Mixing

When creating technical replicates, thoroughly mix the reaction mixture (master mix, sample, and assay) by pipetting the reaction mixture up and down ten times, using 90% volume strokes. Alternatively, pulse vortex the reaction mixture for 15 sec followed by spinning the sample down. Do not assemble or mix reaction mixtures in the DG8 Cartridge. Figure 10.6 shows replicates that were not sufficiently mixed before droplet generation.

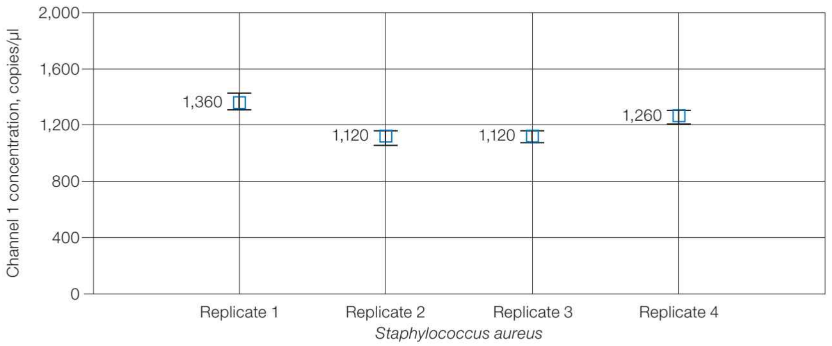


**Fig. 10.6. Quadruplicate replicates drawn from the same poorly mixed reaction solution demonstrate inconsistent concentration readings.**

## Effects of Poor Cycler Uniformity

If the reaction mixtures used to create technical replicates are thoroughly mixed but there is wide variation in concentration estimates, consider the uniformity performance of your thermal cycler. Generally, this effect is observed only when a temperature-sensitive assay is used on a thermal cycler with poor uniformity. Uniformity at both the denaturation and annealing/extension temperatures is important. Bio-Rad's C1000 Touch™ Thermal Cycler with 96–Deep Well Reaction Module has excellent thermal uniformity. To test the module's uniformity, use the temperature-sensitive assay that has concentration variability and create droplets from the same reaction mixture for the entire plate. Check the entire plate for a discrepancy in concentration that exceeds the 95% confidence bounds for the wells. If one of the block's Peltier devices is broken or underperforming, a drop in concentration will be consistently observed in the same quadrant(s) of the block (Figure 10.7).

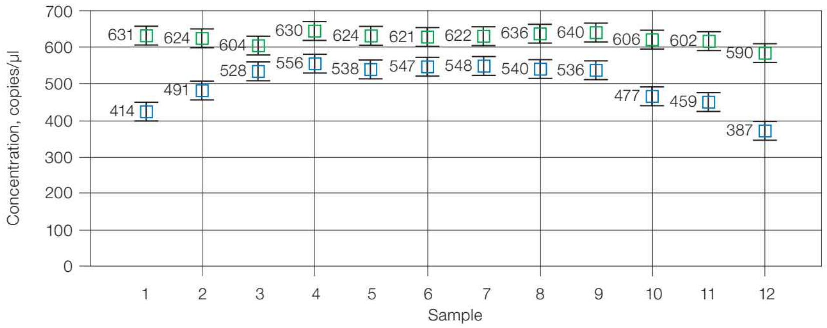


**Fig. 10.7. Concentration plot of a temperature-sensitive assay (🟦) with a temperature-insensitive assay (🟩) concentration across the plate, despite having the same amount of input DNA/well.** All sample wells were loaded with 0.5 copies/droplet Raji DNA.

The temperature-sensitive assay used to generate Figure 10.7 is relatively long and very GC-rich (244 bp, 74% GC). If it is suspected that the variation in concentration is due to thermal cycler performance, consider:

- Increasing the hot start from 94°C for 10 min to 96°C for 10 min
- Raising the denaturation temperature from 94 to 96°C for the first 5 cycles
- Purchasing Bio-Rad's C1000 Touch Thermal Cycler with 96–Deep Well Reaction Module
- If drops in concentration estimates are consistently confined only to a quadrant(s) of the block, contact the manufacturer and request thermal-couple uniformity analysis and, if necessary, repair

## Concentrations Consistently Lower than Predicted

If concentrations measured in ddPCR are consistently lower than predicted, consider poor target accessibility, poor or incorrect assay design, or the presence of PCR inhibitors in samples.

It is possible that the reference concentration measurement that suggests ddPCR concentration calls are low is, in fact, in error and is reporting a higher than actual concentration. ddPCR gives a concentration measurement of intact DNA targets while spectroscopic measurements typically do not distinguish between degraded and intact nucleic acids.

Also consider the following options:

- Make sure the ddPCR assay has been optimized by running a temperature gradient experiment
- Amplicons longer than 150 nucleotides may require longer annealing times during PCR
- If duplexing 2 assays together for the first time, test them in a singleplex assay using the same sample to confirm that the assays are not interfering with one another
- Verify that the fluorophore is not conjugated to a G residue
- Add the recommended primer (900 nM) and probe (250 nM) concentration

# Additional Tips

## No Concentration Calls on Some Wells

If a concentration estimate fails to appear in the concentration chart in QuantaSoft, this indicates the software could not auto-analyze or assign droplets to positive or negative populations using its auto-analysis algorithm, or the well had an unusually low droplet count (<10,000). Low total droplet counts indicate a problem with the assembly of the reaction mix, poor preparation of the sample, or poor handling. Manually set a threshold and QuantaSoft Software will calculate a concentration, which will appear in the concentration chart.

## Target Accessibility

Strong or excessive secondary structure can prevent a DNA target from being amplified. Human gDNA and plasmid DNA can usually be restriction digested to remove inhibiting secondary structure, thereby rescuing detection. RNA secondary structure is best addressed by changing the location of the assay, if possible, or reverse transcribing the assay at a warmer temperature.

Figure 10.8 is an example of poor target accessibility, manifested by the significant number of mid-level amplitude droplets (that is, rain), which is resolved by performing a restriction digestion on the DNA before ddPCR.

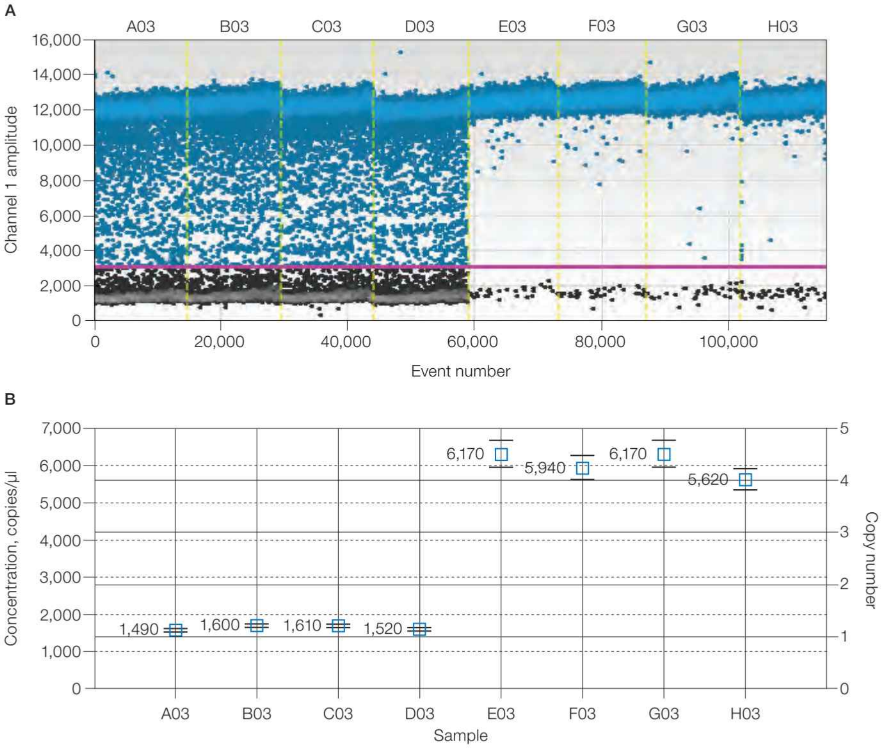


**Fig. 10.8. Elimination of secondary structure allows efficient amplification and accurate quantification.** **A**, fluorescence amplitude plot showing four wells of undigested plasmid DNA (left) and four wells after restriction enzyme digestion to linearize the plasmid (right); **B**, concentrations are corrected to the expected value after restriction enzyme digestion (right) as compared to undigested samples (left).

### High-Fluorescence Amplitude Droplets

Droplet coalescence can create droplets that are much higher in fluorescence amplitude than the other positive droplets. This can be caused by poor droplet transfer technique or extended storage of the droplets pre– or post–thermal cycling. Adjust the scale on the 1-D or 2-D amplitude charts in order to set the thresholds in these cases.

## Troubleshooting EvaGreen® ddPCR Reactions

EvaGreen® binds to both double-stranded DNA and, to a lesser extent, nonspecifically to single-stranded DNA. Therefore, adding excessive amounts of primer or DNA starting materials to EvaGreen® ddPCR reactions can result in an inability to distinguish positive droplets from negative droplets. The separation of positive and negative fluorescence can be affected by total added DNA, including primer concentrations, in addition to the previously discussed fluorescence amplitude differences in amplicon length or amplicons with varying PCR efficiencies.

For good separation, a combined primer concentration between 75–250 nM is recommended and 100–200 nM is optimal (Figures 10.9A–B). Figure 10.9C is an example of the effect of EvaGreen® ddPCR with 900 nM primers on increasing concentrations of DNA that should be negative for the primer set added. Figure 10.9D shows the primer concentration is decreased to 100 nM and the negatives are returned to a uniform cluster of droplets. Similarly, adding more than 130 ng DNA/20 µl reaction can cause the negative droplets and positive droplets to have poor separation. Figure 10.10 shows an experiment with 100 copies/µl mutant DNA spiked into increasing amounts of wild-type DNA. As the total added DNA concentration increases, the positive fluorescence amplitudes decrease and the negative fluorescence amplitudes increase.

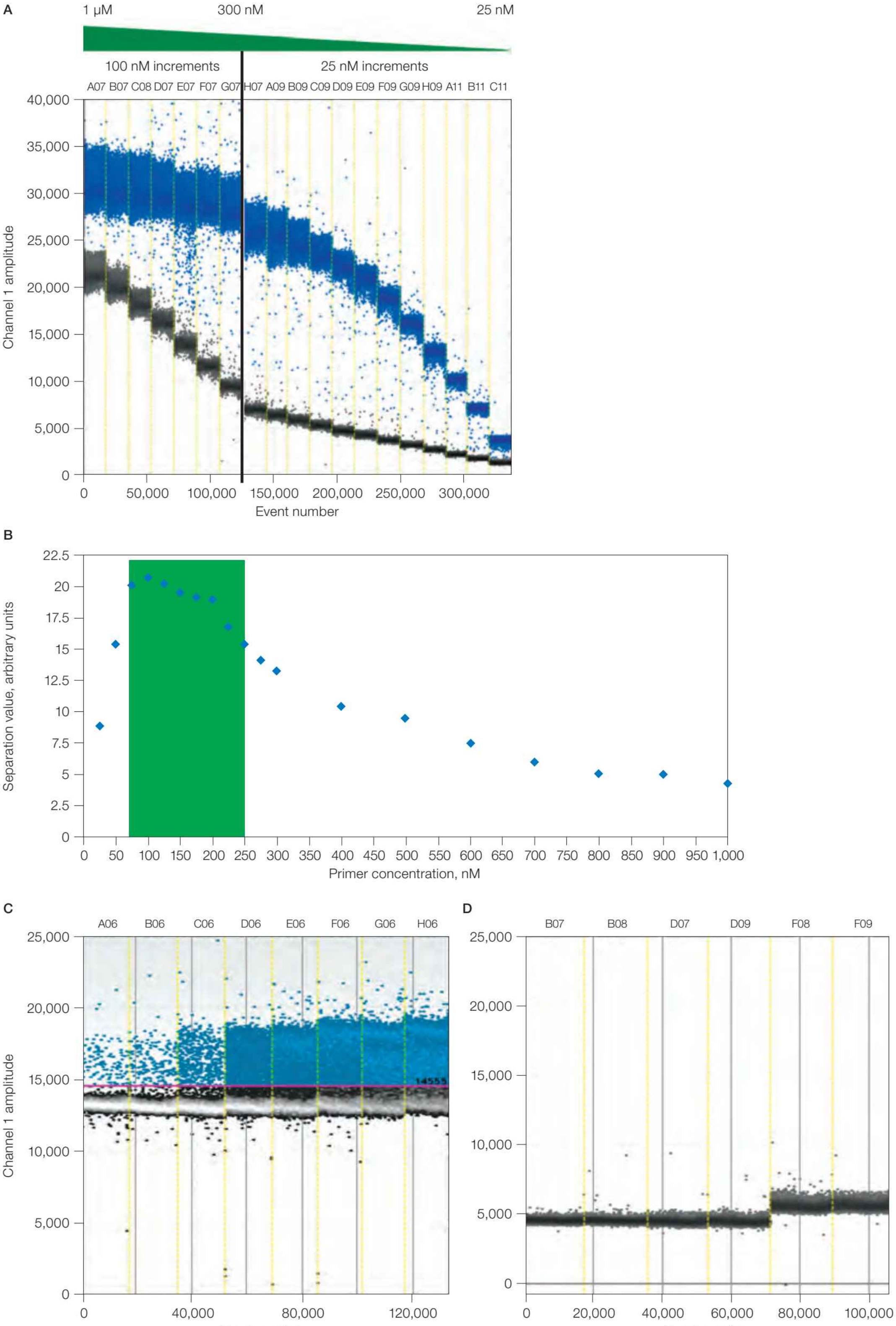


**Fig. 10.9. Good separation of positive and negative droplets in EvaGreen® ddPCR reactions. A–B**, combined primer concentration between 75–250 nM is shown, with 100–200 nM being optimal; **C**, effect of EvaGreen® ddPCR reaction with 900 nM primers on increasing concentrations of DNA; **D**, decreasing the primer concentration to 100 nM produces a uniform cluster of negative droplets.

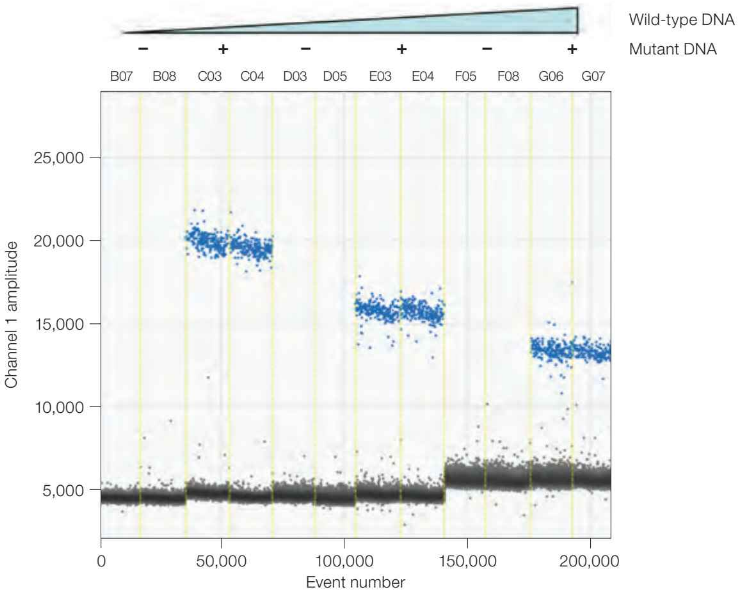


**Fig. 10.10. Mutant DNA spiked into increasing amounts of wild-type DNA.** As the total added DNA concentration increases, the positive fluorescence amplitudes decrease and the negative fluorescence amplitudes increase.

# Reference

Kwok S and Higuchi R (1989). Avoiding false positives with PCR. Nature 339, 237–238.

# Appendix A
# Ordering Information

## Droplet Digital™ PCR (ddPCR™) Systems

### QX200™ Droplet Digital™ PCR Systems

| | |
|---|---|
| 1864001 | **QX200 Droplet Digital PCR System**, includes droplet generator, droplet reader, laptop computer, software, associated component consumables |
| 1864100 | **QX200™ AutoDG™ Droplet Digital PCR System**, includes Automated Droplet Generator, droplet reader, laptop computer |
| 1864002 | **QX200 Droplet Generator**, includes droplet generator, 1 box of 24 cartridges, 1 pkg of 24 gaskets, 2 cartridge holders, 1 power cord |
| 1864101 | **Automated Droplet Generator**, includes Automated Droplet Generator, 1 power cord |
| 1864003 | **QX200 Droplet Reader**, includes droplet reader, 2 plate holders, 1 USB cable, 1 power cord |

## Droplet Digital™ PCR Reagents

### ddPCR Supermix for Probes (No dUTP)

| | |
|---|---|
| 1863023 | **ddPCR Supermix for Probes (no dUTP)**, 2 x 1 ml vials, 2x supermix, for use in nucleic acid sample preparation with the QX100™ or QX200 Droplet Generator |
| 1863024 | **ddPCR Supermix for Probes (no dUTP)**, 5 x 1 ml vials, 2x supermix, for use in nucleic acid sample preparation with the QX100 or QX200 Droplet Generator |
| 1863025 | **ddPCR Supermix for Probes (no dUTP)**, 5 x 5 ml vials, 2x supermix, for use in nucleic acid sample preparation with the QX100 or QX200 Droplet Generator |

### ddPCR Supermix for Probes

| | |
|---|---|
| 1863026 | **ddPCR Supermix for Probes**, 2 x 1 ml vials, 2x supermix, for use in sample preparation with the QX100 or QX200 Droplet Generator |
| 1863010 | **ddPCR Supermix for Probes**, 5 x 1 ml vials, 2x supermix, for use in sample preparation with the QX100 or QX200 Droplet Generator |
| 1863027 | **ddPCR Supermix for Probes**, 5 x 5 ml vials, 2x supermix, for use in sample preparation with the QX100 or QX200 Droplet Generator |
| 1863028 | **ddPCR Supermix for Probes**, 10 x 5 ml vials, 2x supermix, for use in sample preparation with the QX100 or QX200 Droplet Generator |

### ddPCR Supermix for Residual DNA Quantification

| | |
|---|---|
| 1864037 | **ddPCR Supermix for Residual DNA Quantification**, 2 x 1 ml vials, 2x supermix, for use in residual DNA detection with the QX100 or QX200 Droplet Generator |
| 1864038 | **ddPCR Supermix for Residual DNA Quantification**, 5 x 1 ml vials, 2x supermix, for use in residual DNA detection with the QX100 or QX200 Droplet Generator |
| 1864039 | **ddPCR Supermix for Residual DNA Quantification**, 5 x 5 ml vials, 2x supermix, for use in residual DNA detection with the QX100 or QX200 Droplet Generator |
| 1864040 | **ddPCR Supermix for Residual DNA Quantification**, 10 x 5 ml vials, 2x supermix, for use in residual DNA detection with the QX100 or QX200 Droplet Generator |

### One-Step RT-ddPCR Advanced Kit for Probes

| | |
|---|---|
| 1864021 | **One-Step RT-ddPCR Advanced Kit for Probes**, 2 x 1 ml, 200 x 20 µl reactions |
| 1864022 | **One-Step RT-ddPCR Advanced Kit for Probes**, 5 x 1 ml, 500 x 20 µl reactions |

### QX200 ddPCR EvaGreen® Supermix

| | |
|---|---|
| 1864033 | **QX200 ddPCR EvaGreen® Supermix**, 2 x 1 ml vials, for use in nucleic acid sample preparation with the QX200 Droplet Generator |
| 1864034 | **QX200 ddPCR EvaGreen® Supermix**, 5 x 1 ml vials, for use in nucleic acid sample preparation with the QX200 Droplet Generator |
| 1864035 | **QX200 ddPCR EvaGreen® Supermix**, 5 x 5 ml vials, for use in nucleic acid sample preparation with the QX200 Droplet Generator |
| 1864036 | **QX200 ddPCR EvaGreen® Supermix**, 10 x 5 ml vials, for use in nucleic acid sample preparation with the QX200 Droplet Generator |

### ddPCR Mutation Screening Kits

| | |
|---|---|
| 1863506 | **ddPCR *KRAS G12/G13* Screening Kit**, includes 20x multiplex assay and 2x ddPCR supermix for probes (no dUTP) |
| 12001626 | **ddPCR *KRAS Q61* Screening Kit**, includes 20x multiplex assay and 2x ddPCR supermix for probes (no dUTP) |

| | |
|---|---|
| 12001037 | **ddPCR *BRAF V600* Screening Kit**, includes 20x multiplex assay and 2x ddPCR supermix for probes (no dUTP) |
| 12001006 | **ddPCR *NRAS Q61* Screening Kit**, includes 20x multiplex assay and 2x ddPCR supermix for probes (no dUTP) |
| 12001094 | **ddPCR *NRAS G12* Screening Kit**, includes 20x multiplex assay and 2x ddPCR supermix for probes (no dUTP) |
| 12001627 | **ddPCR *NRAS G12/G13* Screening Kit**, includes 20x multiplex assay and 2x ddPCR supermix for probes (no dUTP) |
| 12002392 | **ddPCR *EGFR* Exon 19 Deletions Screening Kit**, includes 20x multiplex assay and 2x ddPCR supermix for probes (no dUTP) |

### ddPCR Residual DNA Quantification Kits

| | |
|---|---|
| 17000031 | **ddPCR CHO Residual DNA Quantification Kit**, includes 20x CHO RDQ assay and 2x ddPCR supermix for residual DNA quantification |
| 17000032 | **ddPCR *E. coli* Residual DNA Quantification Kit**, includes 20x *E. coli* RDQ assay and 2x ddPCR supermix for residual DNA quantification |

### ddPCR *SMN1* Copy Number Determination Kit

| | |
|---|---|
| 1863500 | **ddPCR *SMN1* Copy Number Determination Kit**, includes assay at 20x concentration, 2x ddPCR supermix for probes (no dUTP), and positive controls |

### ddPCR *SMN2* Copy Number Determination Kit

| | |
|---|---|
| 1863503 | **ddPCR *SMN2* Copy Number Determination Kit**, includes assay at 20x concentration, 2x ddPCR supermix for probes (no dUTP), and positive controls |

### ddPCR Library Quantification Kit

| | |
|---|---|
| 1863040 | **ddPCR Library Quantification Kit for Illumina TruSeq**, includes 1 vial of primers and probes at 20x concentration, 2x ddPCR supermix for probes (no dUTP) |

### ddPCR Genome Edit Detection Assays

| | |
|---|---|
| 12002312 | **ddPCR HDR Gene Edit Assay**, 100 rxns |
| 12002313 | **ddPCR HDR Gene Edit Assay**, 500 rxns |
| 12003796 | **ddPCR HDR Gene Edit Pkg**, 1,000 rxns |
| 12003805 | **ddPCR HDR Ref Assay, Predesigned**, 100 rxns |
| 12003806 | **ddPCR HDR Ref Assay, Predesigned**, 500 rxns |
| 12003793 | **ddPCR HDR Ref Pkg, Predesigned**, 1,000 rxns |
| 12002314 | **ddPCR NHEJ Gene Edit Assay**, 100 rxns |
| 12002315 | **ddPCR NHEJ Gene Edit Assay**, 500 rxns |
| 12003794 | **ddPCR NHEJ Gene Edit Pkg**, 1,000 rxns |

# Droplet Digital PCR Consumables and Accessories

### Consumables and Accessories for Both QX100 and QX200 ddPCR Systems

| | |
|---|---|
| 1863004 | **ddPCR Droplet Reader Oil**, 2 x 1 L bottles |
| 1863005 | **Droplet Generation Oil for Probes**, 10 x 7 ml bottles |
| 1864008 | **DG8™ Cartridges for QX100/QX200 Droplet Generator**, 1 pkg of 24 cartridges |
| 1863009 | **DG8 Gaskets for QX100/QX200 Droplet Generator**, 1 pkg of 24 gaskets |
| 1864007 | **Droplet Generator Cartridges and Gaskets**, 5 pkg of 24 DG8 Cartridges, 5 pkg of 24 DG8 Gaskets |
| 1863051 | **DG8 Cartridge Holder** |
| 12001925 | **ddPCR 96-Well Plates**, pkg of 25, clear shell, clear well, semi-skirted plates, for use with QX100, QX200, or QX200 AutoDG Droplet Digital PCR systems |

### Consumables and Accessories for QX200 ddPCR System Only

| | |
|---|---|
| 1864005 | **Droplet Generation Oil for EvaGreen®**, 2 x 7 ml bottles |
| 1864006 | **Droplet Generation Oil for EvaGreen®**, 10 x 7 ml bottles |

### Consumables and Accessories for Automated Droplet Generator Only

| | |
|---|---|
| 11864108 | **DG32™ Automated Droplet Generator Cartridges**, 1 pkg of 30, enough for 10 x 96-well ddPCR plates |
| 1864109 | **DG32 Automated Droplet Generator Cartridges**, 1 pkg of 60, enough for 20 x 96-well ddPCR plates |
| 1864110 | **Automated Droplet Generation Oil for Probes**, 140 ml, enough for 20 x 96-well ddPCR plates |
| 1864112 | **Automated Droplet Generation Oil for EvaGreen®**, 140 ml, enough for 20 x 96-well ddPCR plates |
| 1864120 | **Pipet Tips for AutoDG System**, 1 pkg of 20, enough for 10 x 96-well ddPCR plates |
| 1864121 | **Pipet Tips for AutoDG System**, 1 pkg of 40, enough for 20 x 96-well ddPCR plates |
| 1864125 | **Pipet Tip Waste Bins for AutoDG System**, 1 pkg of 10, enough for 10 x 96-well ddPCR plates |

# Appendix B
# Technical Error Bars in Droplet Digital™ PCR

Because Droplet Digital PCR (ddPCR™) is a digital counting system, you can calculate the measurement error based on the droplet data from a single well. This is the technical error (also referred to as the Poisson error in QuantaSoft™ Software), and it is a good estimate of the errors you can expect to see on true technical replicates (defined as aliquots of the same starting material loaded into multiple ddPCR wells).

The technical error can be calculated from the data points obtained from a single well or multiple wells merged together. For a good assay, the technical error will be approximately equal to the standard error of the mean.

Conceptually, there are two contributions to the error bars: subsampling and partitioning.

## Subsampling

In most molecular biology experiments, we analyze part of a whole (a subsample). Examples include:

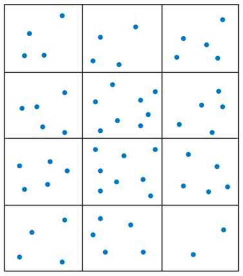
- Blood sample
- Tumor biopsy
- Aliquot from a tube of DNA

Whenever you subsample from a larger volume with the intent to measure properties of the whole volume, random effects will lead to slightly different measurements from the subsampled volume. Subsampling error is most significant at low concentrations. While some quantification systems do not directly report the subsampling error, the standard error of the mean (the typical error reported for replicates) implicitly combines all the different sources of error, including the subsampling error.

**Subsampling example:** Suppose a person has a total of 100,000 copies of a particular target in his or her blood (5 L total volume) and you take 5 ml of plasma. On average, this 5 ml will contain 100 copies of target. But if you take 100 x 5 ml samples, about 16% of them will contain less than 90 copies and about 16% of them will contain more than 110 copies of target. This type of variability is inherent in any type of subsampling.

Figure 1 illustrates subsampling error that arises because a small volume was taken from a large amount of starting material. If the 12 µl of sample has 5 target molecules/µl and you take 1 µl of the sample (1/12th of the whole) and count the number of molecules in that subsample, you may expect to see 5 molecules. However, it is unlikely that you'll see exactly 5. Instead, you might see 3, 4, 6, 7, or 8 molecules. This uncertainty is what we mean by the subsampling error.

Subsampling statistics provide a lower limit on the measurement error, completely independent of the instrument used for measurement. Any additional error introduced by instrumentation would be added on top of this error. In ddPCR in the low concentration regime, this unavoidable subsampling error is the most significant source of measurement error. More droplets will not change this error: 200 targets in 20,000 droplets and 200 targets in 100,000 droplets (five wells) will lead to the same subsampling error.



Count molecules in a subsample (1/12th shown here).

60 molecules in sample. Subsample 1/12 (5 molecules expected).

Most of the 25 subsamples contain 4, 5, or 6 molecules — this uncertainty is what we mean by subsampling error.

**Fig. 1. Subsampling error due to analyzing part of a larger whole.**

The error due to subsampling is given as:

$$\text{Standard deviation} = \sqrt{M}$$

$$\text{Coefficient of variation} = \frac{\sqrt{M}}{M}$$

where $M$ = expected number of target molecules in the ddPCR reaction.

# Partitioning

The second contribution to the technical error bars comes from partitioning of the DNA targets into droplets. This is the error that dominates at high concentration.

The illustration in Figure 2 shows 288 target molecules partitioned into 144 droplets. These grids are snapshots in time: imagine molecules bouncing around in the sample, then at any given instant divide the sample into 288 partitions. It's evident that most of the partitions do not contain exactly two molecules.

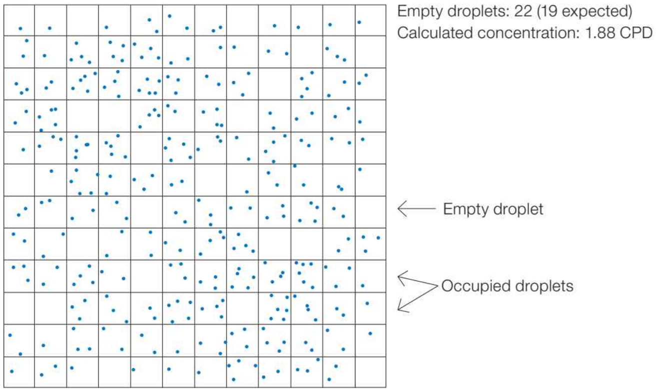

Empty droplets: 22 (19 expected)
Calculated concentration: 1.88 CPD
Empty droplet
Occupied droplets

**Fig. 2. Target molecule partitioning.** CPD, copies per droplet.

Statistics tells us that we should on average see 19 (or more precisely, 19.5) empty partitions or droplets in this experiment. In a particular instant in time, we see 22 empty droplets and we estimate a concentration of 1.88 based on this number. The partitioning in Figure 3 shows different instants in time, and correspondingly different numbers of empty droplets (note that the empty "droplets" are highlighted by a thicker black line). This type of error is governed by Poisson statistics, just like the subsampling error, and is referred to as the "partitioning error."

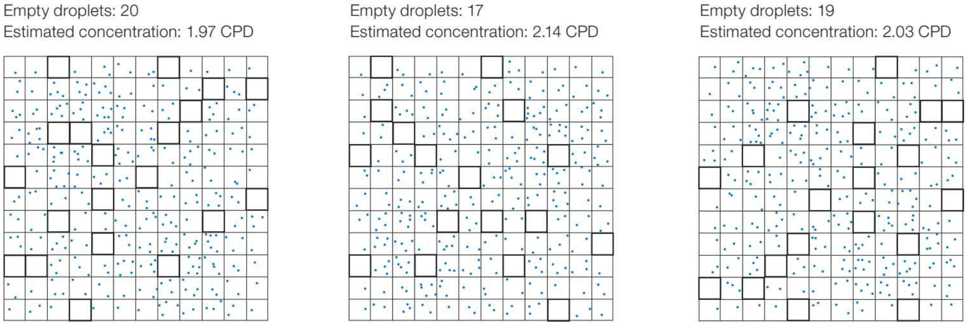

Empty droplets: 20
Estimated concentration: 1.97 CPD
Empty droplets: 17
Estimated concentration: 2.14 CPD
Empty droplets: 19
Estimated concentration: 2.03 CPD

**Fig. 3. Target molecule partitioning repeated three times.** CPD, copies per droplet.

Figure 4 illustrates the technical error as a function of CPD for 10,000 droplets. Note that the coefficient of variation (CV) is extremely low across the entire range covered here. The dotted lines show the region with CV <2.5%: 0.17–5.1 CPD.

The black and blue curves match very closely at low CPD, meaning that subsampling error is more significant than the partitioning error in this example. The blue curve goes up with high CPD as the contribution of partitioning error increases.

**Note:** The actual CV calculations contain a Bayesian adjustment at very low and very high CPD (<100 occupied or <100 empty droplets).

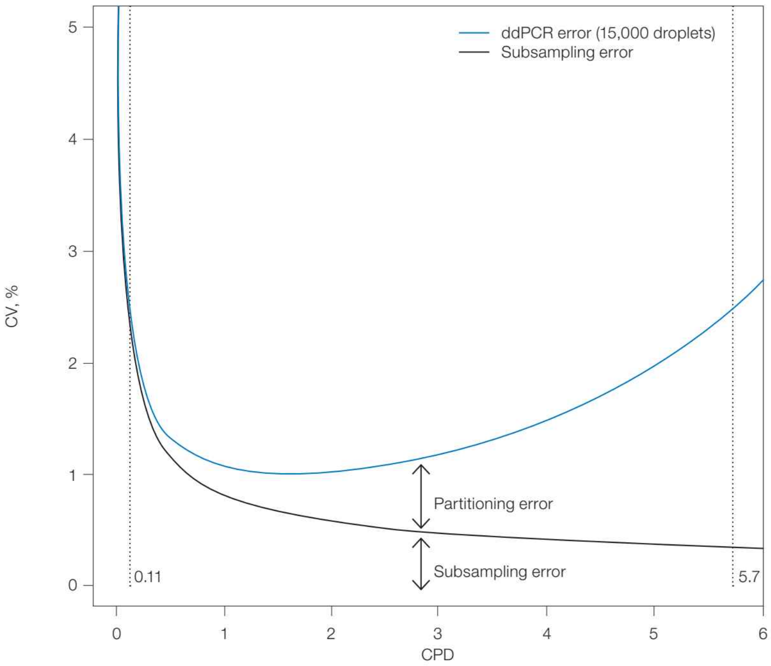


**Fig. 4. Relative contribution of partitioning and subsampling errors to ddPCR error.** CPD, copies per droplet; CV, coefficient of variation.

# Appendix C
# Acronyms

## Acronyms

ABS — absolute quantification

BLAST — Basic Local Alignment Search Tool

bp — base pair

cDNA — complementary DNA

CN — copy number

CNV — copy number variation

CPD — copies per droplet

CV — coefficient of variation

ddPCR™ — Droplet Digital™ PCR

DNA — deoxyribonucleic acid

dsDNA — double-stranded DNA

dUTP — 2'-deoxyuridine 5'-triphosphate

*ERBB2* — human epidermal growth factor receptor 2 gene (also, *HER2*)

FFPE —formalin-fixed, paraffin-embedded

FISH — fluorescence in situ hybridization

GC — guanine-cytosine

gDNA — genomic DNA

GMO — genetically modified organism

*HER2* — human epidermal growth factor receptor 2 gene (also, *ERBB2*)

HIV — human immunodeficiency virus

IHC — immunohistochemistry

LNA — locked nucleic acid

LoD — limit of detection

LoQ — limit of quantification

miRNA — microRNA

NGS — next-generation sequencing

NTC — no template control

PBMC — peripheral blood mononuclear cell

PCR — polymerase chain reaction

qPCR — quantitative PCR

RED — rare target sequence detection (rare event detection)

RFU — relative fluorescence units

RMD — rare mutation detection

RNA — ribonucleic acid

RSD — rare sequence detection

RT-ddPCR — reverse transcription Droplet Digital PCR

RT-qPCR — reverse transcription quantitative PCR

SNP — single nucleotide polymorphism

$T_M$ — melting temperature

UDG — uracil DNA glycosylase

UNG — uracil N-glycosylase

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